Genetic variability in the pistachio late blight fungus, *Alternaria alternata*

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Genetic variation in the pistachio late blight fungus, *Alternaria alternata*, was investigated by restriction fragment length polymorphism (RFLP) in the rDNA region. Southern hybridization of *Eco*RI, *Hin*dIII, and *Xba*I digested fungal DNA with a RNA probe derived from Alt1, an rDNA clone isolated from a genomic library of the Japanese pear pathotype of *A. alternata*, revealed 34 different rDNA haplotypes among 56 isolates collected from four central valley locations in California. Analysis of molecular variation revealed a significant amount of genetic diversity within populations (85.8%), with only marginal variation accounting for differentiation among populations (14.2%, $\Phi_{ST} = 0.142$). All isolates examined were highly pathogenic. The identity of the four geographic populations sampled was not evident in both cluster and principal component analyses, probably indicating either the selectively neutral nature of rDNA variation or prevalence of widespread gene flow among populations combined with uniform host-selection.

INTRODUCTION

The genus *Alternaria* contains diverse and ubiquitous species of fungi, including aggressive and opportunistic plant pathogens affecting the majority of cultivated plants. One of the best known and economically important members of the genus is *A. alternata*, causal agent of late blight on California pistachios (*Pistacia vera*). *A. alternata* is distributed throughout the pistachio growing areas of California with latent infections occurring on leaves and fruits early in the summer, becoming severe during fruit development and maturity in late July and early August (Michailides *et al.* 1998). Under favourable conditions, the disease can cause severe defoliation, and deteriorates nut quality and appearance by infecting kernels in early splits and staining shells, thus reducing marketable yields (Evans, Michailides & Morgan 1997). The fungus can be readily isolated from lesions on leaves and cultured on potato-dextrose agar (PDA) or potato-carrot agar (PCA). On PDA, it produces fluffy dull greenish-gray aerial mycelium with small darkly pigmented conidia borne in short chains, while on PCA, it produces a thin mat of mycelium with abundant spores after 3–4 d of incubation at 24 °C under light.

As a pathogen, *A. alternata* often exhibits variation among field isolates for toxin production, degree of pathogenicity, and symptom expression, suggesting the existence of multiple biotypes within the species (Adachi *et al.* 1993, Weir *et al.* 1998). Biochemical and molecular markers have been used to demonstrate the existence of multiple strains and to assess infraspecific variation in *A. alternata* at the protein and DNA level (Petrunak & Christ 1992, Adachi *et al.* 1993, Weir *et al.* 1998). Petrunak & Christ (1992) assessed isozyme variation in *A. alternata* and delineated 23 electrophoretic types from among 96 isolates assayed from various hosts and geographic locations. Adachi *et al.* (1993) demonstrated RFLPs in the rDNA region on both a micro- and macrogeographical scale among isolates of the Japanese pear pathotype of *A. alternata* and grouped 271 isolates into eight rDNA types.

Genetic analysis of plant pathogen populations is fundamental to the understanding of epidemiology, host–pathogen coevolution, and resistance management (McDonald *et al.* 1989, Leung, Nelson & Leach 1993, Milgroom & Fry 1997). Restriction fragment length polymorphisms (RFLPs) have been used extensively in analyzing genetic variability and structure in natural populations of plant pathogenic fungi (Michelmore & Hulbert 1987, McDonald *et al.* 1989, Bruns, White & Taylor 1991, Milgroom, Lipari & Powell 1992, Adachi *et al.* 1993, Weir *et al.* 1998). Knowledge of pathogen variation and its spatio-temporal distribution allows for more accurate assessment of resistant germplasm and breeding populations, and also aids in developing strategies to deploy resistant genotypes in space and time that promote stable pathogen populations and delay development of biotypes that overcome host resistance (Leung *et al.* 1993). This is especially important in tree crops, where host resistance must

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endure for several decades during the productive life-span of trees.

The objectives of the present study were to: (1) fingerprint and evaluate within population genetic diversity; and (2) examine the genetic differentiation within and between populations from diverse pistachio-growing environments in central valley California. Alt1, an rDNA clone isolated from the genomic library of the Japanese pear pathotype of *A. alternata*, kindly supplied by Y. Adachi (Nagoya University, Japan) was used to characterize the *A. alternata* isolates from pistachio. Alt1 has been well characterized and restriction mapped, and is known to contain two copies of the entire rDNA unit (Tsuge, Kobayashi & Nishimura 1989).

MATERIALS AND METHODS

Collection and culture of fungal isolates

Field isolates of *Alternaria alternata* were obtained from diseased leaves collected from four well isolated locations in California: Wolfskill Experimental Orchard (WEO) near Sacramento; Kearney Agricultural Center (KAC), south of Fresno; two pistachio breeding plots, one each from near Bakersfield (BAK-E) and Kettleman City (BAK-W) (Table 1, Fig. 1). These locations represent diverse pistachio-growing environments in California, where annual cycles of *Alternaria* infection are common. At each location, 15–20 randomly selected trees from an area of approximately 2–3 ha were sampled for diseased leaves. Leaves were washed in distilled water and rinsed in sterile water before excising discrete lesions into about 4-mm² pieces with a sterile blade. A single sample was removed from each leaf although many lesions were found on the same leaf. The pieces were rinsed once again in sterile water, and placed on moist filter paper in sterile petri dishes and incubated at 28 °C for 2 d. Conidia from the lesion surface were spread onto 3% water agar in a petri plate with a sterile loop and incubated overnight at 28 °. Single germinating spores were isolated and transferred to potato-carrot agar (PCA) (Dhingra & Sinclair 1986). A total of 56 isolates from the four locations/populations (14 per population) were analyzed. Isolates were stored on PCA slants in a refrigerator for future use and reference, and representative strains deposited in the American Type Culture Collection (ATCC).

Assay for pathogenicity

Each isolate was cultured on a PCA plate at 28 ° for 5 d under light. Small circular cotton swabs approx. 0.5 cm diam soaked in sterile water were gently rubbed on the cultures to collect significant spore populations. The moist swabs with spores were applied firmly on to the undersurface of leaves of the pistachio cultivar 'Kerman' after slightly wounding the leaf at the center with a sterile needle under field conditions. 'Kerman' is the only female cultivar used in the commercial production of pistachios in California. Strips of cellophane tape were used to hold the cotton swabs in place during incubation. Care was exercised to avoid cross contamination by selecting well isolated leaves and restricting one isolate per leaf during inoculation and incubation. Thick transparent plastic bags enclosed within brown paper bags were used to cover the entire branch during incubation, and the outer

Table 1. Origin and pathogenicity of the *Alternaria alternata* isolates used in the study.

Isolate	Origin*	Pathogenicity†	
		Day 5	Day 7
K2-3	KAC	5	5
K1-3	KAC		5
K1-4	KAC	2	5
K3-4	KAC	4	5
K3-3	KAC	0	3
K3-2	KAC	4	5
K2-5	KAC	5	5
K2-2	KAC	3	5
K1-5	KAC	2	3
K2-1	KAC	3	5
K2-4	KAC	5	5
K2-3-1	KAC		5
W2-1	WEO	5	5
W3-2	WEO	5	5
W4-2-2	WEO	4	5
W4-3	WEO		3
W2-4	WEO	2	5
W3-3	WEO	2	5
W3-1	WEO	5	5
W4-1	WEO	5	5
W2-3	WEO	5	5
W2-2	WEO	5	5
W4-2-1	WEO		4
W3-3-1	WEO	1	2
W4-1	WEO	5	5
W3-4	WEO	5	5
BE2-2	BAK-E	5	5
BE2-3	BAK-E		3
BE1-2	BAK-E	4	5
BE1-1	BAK-E	2	4
BE3-1	BAK-E	3	5
BE2-1	BAK-E		5
BE1-3	BAK-E	3	4
BE3-2	BAK-E	3	4
BE3-3	BAK-E	5	5
BE4-1	BAK-E	4	5
BE2-4	BAK-E	4	5
BE3-2	BAK-E		4
BE2-2-1	BAK-E	3	5
BE5-2	BAK-E	2	3
BW2-2-2	BAK-W		1
BW3-4	BAK-W	0	4
BE2-2-1	BAK-W	3	5
BW3-3	BAK-W	5	5
BW2-3	BAK-W	4	4
BW3-6	BAK-W		4
BW3-2	BAK-W	2	3
BW3-1	BAK-W	5	5
BW1-5	BAK-W	5	5
BW2-1	BAK-W	4	5
BW1-3	BAK-W	3	5
BW1-2	BAK-W	5	5
BW1-1	BAK-W		5
BW1-4	BAK-W		3

* KAC, Kearney Agricultural Center, south of Fresno, CA; WEO, Wolfskill Experimental Orchard, near Sacramento, CA; BAK-E and BAK-W, pistachio breeding plots near Bakersfield and Kettleman city, CA respectively.

† Pathogenicity scored on days 5 and 7 after inoculation and in a 1–5 scale where 1 is the least virulent.

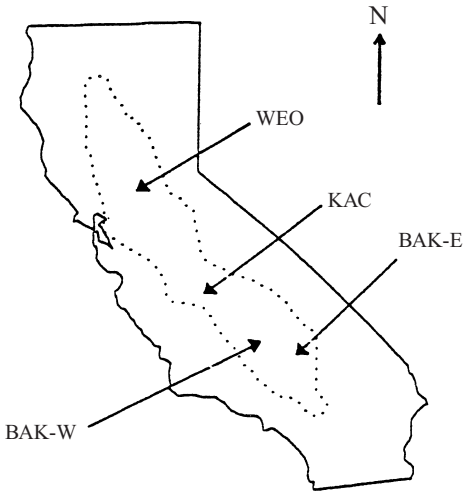


Fig. 1. Sampling locations of field isolates of pistachio late blight fungus, *Alternaria alternata*, representing diverse pistachio growing environments in California.

brown paper bags were removed after 48 h. After 5 and 7 days of inoculation, pathogenicity was evaluated based on the size, shape, and sporulation in the necrotic spots on a 1–5 scale with the least infective being scored as unity and the most infective as five.

DNA extraction and Southern hybridization with RNA probe

Cultures were grown in 100 ml of sterile glucose-asparagine broth in 250 ml conical flasks for 4–5 d on a rotary shaker at 120 rpm. The composition and final concentrations of the broth (l^{-1}) were as follows: 1.2 g L-asparagine; 100 mg NaCl; 1.2 g $K_2HPO_4 . 3H_2O$; 500 mg $MgSO_4 . 7H_2O$; 500 mg yeast extract; 20.7 g glucose. Mycelium was harvested by vacuum filtering through Whatman No. 1 filter paper and approximately 1.5 g of mycelium was ground in liquid nitrogen with a mortar and pestle. Powdered mycelium was quickly dispersed in 8 ml of hot 4% CTAB extraction buffer (Doyle & Doyle 1987) containing 50 µl of β-mercaptoethanol, in a 50 ml tube, and incubated at 65 ° for 30 min followed by extraction with 8 ml of chloroform/isoamyl alcohol (24:1, v/v) for 15 min and centrifugation at 10000 rpm. The supernatant was extracted with equal volumes of phenol/chloroform (1:1, v/v) and chloroform/isoamyl alcohol (24:1, v/v). Nucleic acids were precipitated with one volume of isopropanol and centrifuged to pellet the DNA. The pellet was washed twice with 75% ethanol containing 10 mM ammonium acetate, air-dried, and dissolved in 500 µl of TE buffer and treated with 20 µg of RNase A (Sigma) per ml at 37 ° for 1 h.

Fungal DNA was digested with the restriction enzymes, *Alu*I, *Eco*RI, *Hin*dIII, *Hin*fI, and *Xba*I for 2–4 h according to the manufacturer's instruction (Stratagene, La Jolla, CA). Digested DNA (approx. 1.5 µg per lane) along with nonradioactively labelled λ *Hin*dIII size standard were separated in a 1 cm thick 0.8% agarose gel prepared with 1 × TBE buffer by running for 20 h at 1.0 V per cm. Upon completion of electrophoresis, gels were stained briefly with ethidium bromide and photographed. Capillary transfer of DNA to nylon$^+$ membrane (Boehringer Mannheim, IN) was conducted overnight (Sambrook, Fritsch & Maniatis 1989).

An *Eco*RI-digested fragment of a λ Fix clone (Stratagene), Alt1, was used as template to produce an RNA probe, as the entire insert (16.9 kb) is too large for *in vitro* RNA synthesis. As per Tsuge *et al.* (1989), the fungal DNA insert within the Alt1 contained four *Eco*RI sites and allows for production of a smaller RNA probe using the fragment adjacent to the flanking T3 primer site in the vector, which includes a major portion of 28S rDNA, the internal transcribed spacer region and a segment of 5.8S rDNA within which the *Eco*RI site is present (Adachi *et al.* 1993: Fig. 3). Nonradioactive RNA labelling and detection kits (Boehringer Mannheim) were used to produce the labelled RNA probe for Southern hybridization and detection of specific sequences according to the manufacturer's protocols. The nonradioactive RNA labelling allows for labelling RNA with digoxigenin-UTP by *in vitro* transcription in the presence of T3 RNA polymerase. Blots were exposed to Kodak X-Omat AR film at room temperature for 15 min.

Data analysis

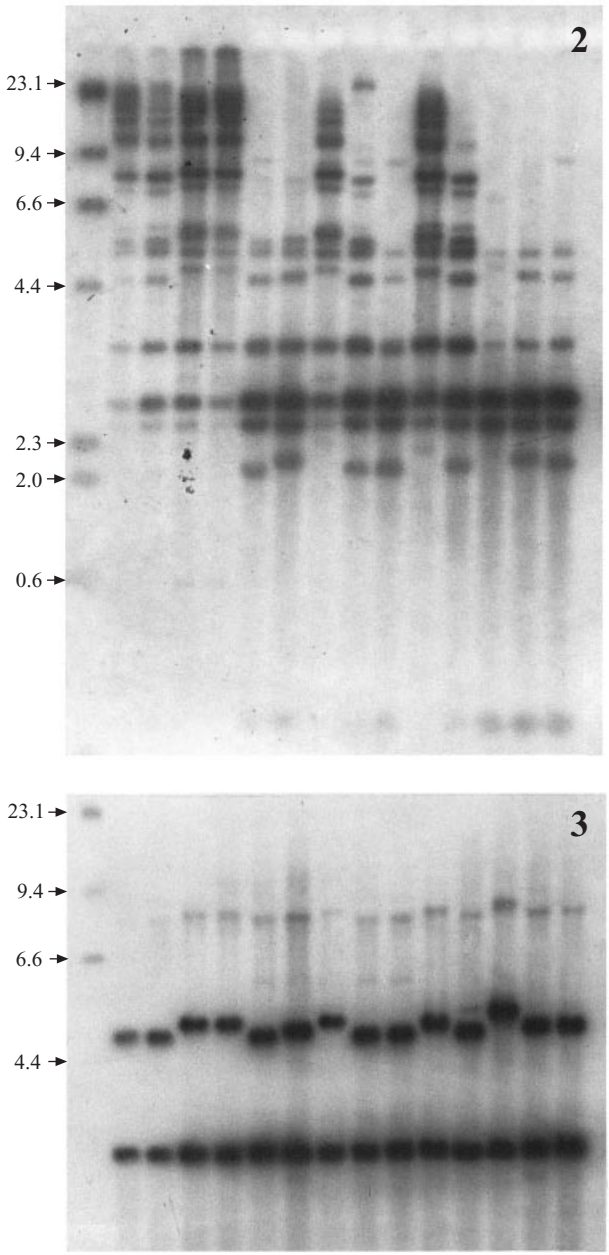
The autoradiographs were scored in binary format with band presence scored as unity and absence as zero for all corresponding positions of bands across all isolates and populations. Analysis of molecular variance was performed using the computer program AMOVA version 1.5 (Excoffier, Smouse & Quattro 1992), which incorporates DNA haplotype divergence into an analysis of variance format. Total variance was partitioned into variance due to within and between populations, and Bartlett's heteroscedasticity statistic was computed to test the heterogeneity of variance among populations (Bartlett 1937). The variance components from the analysis were used to estimate the population subdivision, here referred to as Φ_{ST}, which is the correlation of random pairs of haplotypes drawn from within subpopulations relative to the correlation of pairs of haplotypes drawn from the whole population. Genetic relationship among isolates was computed based on the proportion of DNA fragments shared between two isolates for all possible pair-wise comparisons using equation (21) of Nei & Li (1979). The resulting distance matrix was subjected to a cluster analysis (CA) following the UPGMA algorithm (Sneath & Sokal 1973). The cophenetic correlation coefficient (Rohlf & Sokal, 1981) between the cophenetic matrix and distance matrix was computed to test the goodness of fit of the cluster analysis to the data. Bootstrap analysis (500 replicates) were performed to assess the relative support for different groups using the WinBoot computer program (Yap & Nelson 1996). A two-dimensional (2D) visualization of rDNA variation among isolates along the first two principal axes was obtained by principal component analysis (PCA) to supplement the CA results. Minimum spanning tree (MST) generated from the correlation matrix (Gower & Ross 1969) was superimposed onto the PCA plot to determine how well the original distances were preserved in the 2D PCA plot.

RESULTS AND DISCUSSION

Genetic variability and differentiation within and among populations

Extensive genetic variability in the rDNA region was observed among isolates from different pistachio-growing areas of California. Among the five restriction enzymes used in the digestion of fungal DNA and Southern hybridization with the Alt1-generated RNA probe, *Alu*I and *Hin*fI did not show any variation. The remaining three enzymes, *Eco*RI, *Hin*dIII, and *Xba*I revealed variation among isolates, with *Xba*I generating the largest amount of variation with 22 rDNA haplotypes, followed by *Eco*RI and *Hin*dIII with nine and five haplotypes respectively. A more complex rDNA pattern of variation was observed for *Xba*I with as many as 37 different fragments across isolates from different locations, ranging from 2.0 to 24 kb (Fig. 2), compared to the simple variation for *Eco*RI (9 different fragments; Fig. 3) and *Hin*dIII (10 different fragments). Thirty four different rDNA haplotypes were obtained across all the three restriction enzymes which produced RFLPs among isolates. Earlier studies on *A. alternata* using isozyme markers found considerable variation among isolates (Petrunak & Christ 1992, Weir *et al*. 1998). They suggested natural mutations, abundant sporulation, and parasexual recombination as the possible causes for such high levels of variation. Although it is uncertain whether a teleomorph occurs in nature, formation of heterokaryons in laboratory experiments has been reported in this fungus (Tsuge, Hayashi & Nishimura 1987, Stovall 1992). Further, Adachi & Tsuge (1994) demonstrated in the Japanese pear pathotype of *A. alternata* that genetic recombination, either through asexual or sexual means, can occur during co-infection by genetically different isolates leading to heterogeneity within the rDNA cluster. It is therefore apparent from the high levels of variation reported here and from the earlier studies that this haploid imperfect fungus is capable of recombining natural mutations either through the asexual or sexual cycle to generate diversity in asexual populations.

Hierarchical analysis of molecular variance showed marginal differentiation among populations (14.2%, $\Phi_{ST} = 0.142$), with a large fraction of the total variation residing within populations (85.8%) (Table 2). Xia *et al*. (1993) reported similar results with *Magnaporthe grisea* on rice, where they found 94% of the variation harboured within populations and only a small component partitioned among locations (11%) and between the two fields (3%) examined. Genetic variability studies in the Japanese pear pathotype of *A. alternata* gave somewhat similar results, where only rare rDNA variants contributed to population differentiation (Adachi *et al*. 1993). The within-population component of variance for different populations and Bartlett's test for homogeneity of variances suggest that fungal populations from the different pistachio-growing areas in California are highly variable and possess similar amounts and pattern of variation. Leung & Williams (1986) have suggested that in fungi with a high reproductive capacity, mutations could produce significant DNA variation if the mutations are neutral with respect to fitness. The field inoculation of different isolates on 'Kerman' to assess pathogenicity showed little differences among isolates and most registered a score of 5 (highly pathogenic) on a 1–5 scale (Table 1). Lack of significant geographic differentiation among the *Alternaria* populations studied suggests two possibilities: (1) widespread dispersal of spores (migration) of the fungus by wind and by various biotic and abiotic factors, resulting in extensive gene flow between populations; and (2) the saprophytic nature of *A. alternata* coupled with weak host selection pressure within and between populations. The influence of host selection was stressed as an important factor in determining the population structure and diversity in fungal pathogens (Petrunak & Christ 1992, Xia *et al*. 1993, Weir *et al*.



Figs 2–3. Southern hybridization of *Xba*I and (**Fig. 2**) and *Eco*RI (**Fig. 3**) digested DNA of the pistachio late blight fungus with nonradioactively labelled RNA probe derived from Alt1, an rDNA clone isolated from the Japanese pear pathotype of *Alternaria alternata*. Lanes from left to right: 1, *Hin*dIII digested λ DNA (fragment sizes in kb are shown to the left); 2–15, isolates representing Bakersfield (BAK-E) population.

Table 2. Partitioning of molecular variation into variation due to within and between components.

Source of variation	Variance*	% total	*P*†	Φ-statistics
WEO	1.836			
KAC	1.208			
BAK-E	1.555			
BAK-W	1.746			
Among populations	0.263	14.2	0.002	0.142
Within populations	1.585	85.8	0.002	

* Bartlett's test for heterogeneity of variances among populations = 0.583 (χ^2, c.f. = 3).

† Probability of having a more extreme variance component.

1998). However, further studies with a larger sampling base are needed to develop a full understanding of genetic diversity and differentiation in this ubiquitous fungus.

Genetic relationships among isolates

The UPGMA cluster analysis based on pair-wise genetic similarity coefficients revealed three genetic groups among the isolates at about 60% level of similarity, supported by moderate bootstrap confidence levels (Fig. 4). The identity of geographic populations was not evident in the cluster analysis indicating either selective neutrality of the rDNA variation or homogenizing effects of gene flow between populations coupled with somewhat uniform host-selection. All the three clusters contained isolates from all four populations included in the study. However, the overall composition of different groups in the cluster analysis allows for some generalizations. Isolates originating in WEO and KAC predominantly constituted group 1. Group 2 was predominantly made up of isolates from BAK-E & W populations, except for two isolates from KAC. Group 3 appeared to be an admixture of isolates from KAC, WEO, and BAK-E and may represent isolates with wider distribution and broader adaptation to the growing conditions in the valley as compared to isolates in groups 1 and 2. The two isolates, BW2-2-2 and BW1-3 from BAK-W population, one each in groups 1 and 3, were somewhat genetically distinct from the rest of the isolates within these groups. However, the overall genetic affinities among isolates within and between groups and the clear absence of WEO isolates and presence of only two isolates from KAC in group 2 support marginal differentiation among these populations, corroborating the results of hierarchical analysis of molecular variance. Isolates from the northern and central parts of the

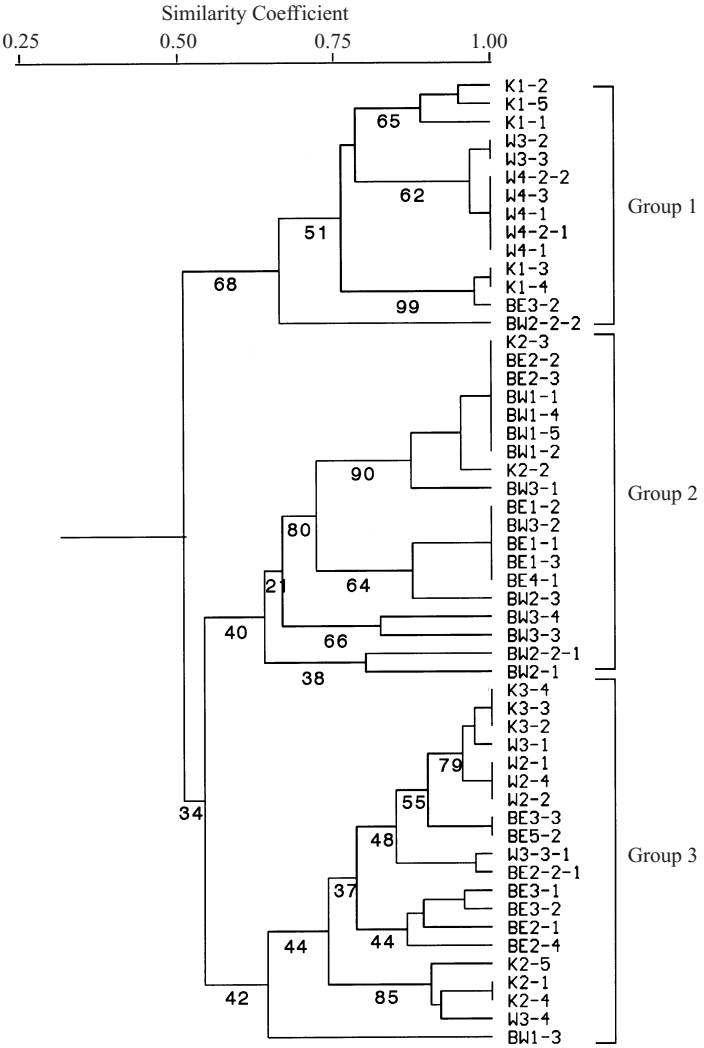


Fig. 4. Dendrogram showing genetic relationships among isolates of pistachio late blight fungus, *Alternaria alternata* from four central valley locations in California. Numbers below branches are bootstrap percentages. (Cophenetic correlation coefficient = 0.856.)

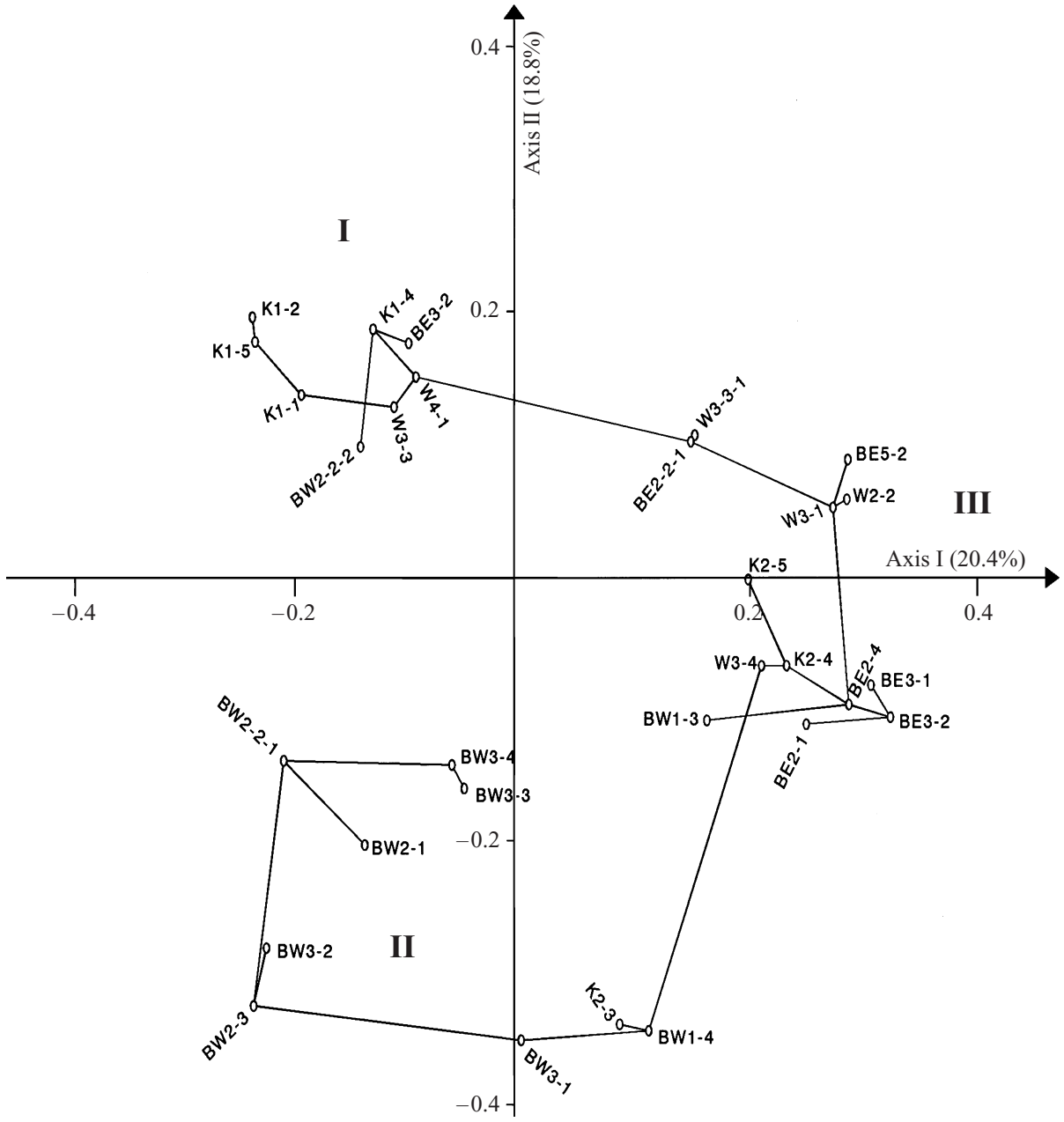


Fig. 5. Two-dimensional projection of *Alternaria alternata* isolates from four central valley locations in California along the first two principal axes with minimum spanning tree superimposed (some isolates overlap).

valley appear to be somewhat distinct from southern valley isolates with some distributed over wider geographic areas. The 2D projection of isolates along the first two principal axes, which accounted for 20.4 and 18.8% of the total variation respectively, revealed three groups in accordance with the results of CA (Fig. 5). Although accounting for only 39.2% of the total variation, the 2D projection resolved the isolates into groups without much distortion. The minimum spanning network superimposed on the PCA plot supported the genetic relationships within and between different groups of isolates and showed that the original proximities were preserved in the 2D projection of isolates.

Coexistence of several strains of *A. alternata* in each of the locations is evident from the rDNA variability study. The *Alternaria* populations sampled are highly variable and have not developed a well defined genetic structure. The saprophytic nature of this fungus coupled with a high reproductive rate and widespread dispersal of spores has enabled it to accumulate and circulate high levels of genetic variability within and between populations. It is apparent from the aforementioned results that: (1) *A. alternata*, with its enormous potential for reproduction and dispersal, and the genetic uniformity of the host, may continue to pose a significant threat to the production of quality pistachios; and (2) favourable demographic and genetic factors may confer adaptive advantage to the fungus to develop novel mechanisms to overcome susceptibility to existing control measures. Development of diverse sources of host resistance, strategic deployment of resistant genotypes, and monitoring of spatio-temporal diversity of pathogen populations should be the major focus of the industry for sustainable production of pistachio in California. Additional studies on a larger sample

base are planned to develop a comprehensive picture of genetic diversity and structure for this pathogen.

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