Genetic Variability in the Potato Pathogen *Colletotrichum coccodes* as Determined by Amplified Fragment Length Polymorphism and Vegetative Compatibility Group Analyses

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ABSTRACT

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Amplified fragment length polymorphism (AFLP) using three primer sets was used to characterize 211 *Colletotrichum coccodes* isolates from North America, 112 of which were assigned to six vegetative compatibility groups (VCGs) using nitrate nonutilizing (*nit*) mutants. These isolates clustered into five corresponding groups by unweighted pairgroup method with arithmetic means-based cluster analysis of AFLP banding patterns. Isolates of *C. coccodes* belonging to NA-VCG1 and NA-VCG3 were closely related, as were isolates belonging to NA-VCG2 and NA-VCG5. Based on bootstrap analysis of AFLP data, the two isolates originally assigned to NA-VCG4 clustered with isolates belonging to NA-VCG2 and NA-VCG5. *C. coccodes* isolates that clustered with two isolates belonging to NA-VCG6 were the most diverged from other groups, including seven isolates collected from hosts other than potato. As opposed to the bootstrap analysis, a quadratic discriminant analysis (QDA) of AFLP data correctly categorized the two isolates of NA-VCG4. Furthermore, in isolates where VCG determinations had been made, this model correctly classified isolates of all VCGs. QDA classifications were identical to those made by the bootstrap analysis, with the exception of VCG4. Overall, classifications made by the QDA model were strongly correlated ($r = 0.970$, $P < 0.001$) to the VCGs assigned by traditional methods. All 99 *C. coccodes* isolates evaluated only by AFLP also were subjected to QDA, leading to the assignment of a presumptive VCG for each isolate. No isolates of VCG4 or VCG6 were identified by QDA within this population. Symptoms of black dot developed in plants inoculated with isolates collected from both potato and non-potato hosts. However, total yield was not significantly reduced by infection with non-potato isolates. The lack of any additional groups identified by AFLP analysis may be an indicator of a limited level of genetic variation among North American *C. coccodes* isolates. AFLP is a much more efficient technique for subspecific characterization in *C. coccodes* than VCG analysis utilizing *nit* mutants and will provide an effective means by which the population biology of this pathogen can be further investigated worldwide.

Colletotrichum coccodes affects crops in the family *Solanaceae*, including tomato and pepper, and is the causative agent of black dot, a tuber blemish and yield- and quality-reducing disease of potato (17,31). *C. coccodes* has been documented as an economically important soil, seed, and foliar pathogen (15,27,31) in many commercial- and seed-production areas. Black dot is characterized by small black sclerotia on senescing and dead plant tissue, including roots, stems, stolons, and tubers, resulting in yield reduction of up to 30% and decreased tuber quality (2, 26,49). *C. coccodes* also has been identified as a contributor to the potato early dying (PED) complex along with other pathogens, including *Verticillium dahliae, V. albo-atrum*, *Rhizoctonia solani*, and *Erwinia carotovora* spp. (39,50). As is the case with other seedborne potato pathogens, it is believed that infected potato seed tubers introduce the pathogen into noninfested areas (6,27). Sclerotia of *C. coccodes* have been documented to survive up to 8 years in infested soil, making this an important source of inoculum for subsequent potato crops (18). To date, no specific fungicides have been developed to control *C. coccodes*. However, fenpiclonil (41), a mixture of chloramizol sulfate with prochloraz (33), and azoxystrobin (36) have been reported to significantly reduce the severity of black dot. The soil fumigant methyl bromide also has been reported to reduce the incidence of black dot (15).

Genetic characterization of plant pathogen populations has become an important tool in providing information about the genetic diversity among and within species, allowing a more accurate and detailed method of identification (13). Traditionally, fungal species have been defined according to, among other things, morphological characteristics and host range. However, these methods do not completely differentiate many fungal taxa, especially at the subspecific level. The application of biochemical and molecular techniques can clarify the relationships among and within fungal groups, contributing to a better understanding of disease epidemiology.

Vegetative compatibility is a genetically determined ability of fungal isolates within a species to form viable heterokaryons via hyphal anastomosis (32,40). Vegetative compatibility has been identified as a system of subspecific classification in many host–pathogen systems, including *Verticillium* spp. (12), *Fusarium* spp. (8), and *Colletotrichum* spp. (9,37). In asexually reproducing fungi, hyphal anastomosis is possibly the sole means of genetic exchange. Isolates that share the ability to anastomose are classified as belonging to the same vegetative compatibility group (VCG). Isolates within a VCG tend to be more similar genetically than isolates from other VCGs (24,25).

It has been proposed (25) that, in many host–pathogen systems, high variation in virulence and host range is present at the sub-

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specific level, which may impact disease severity and management. *V. dahliae* has been separated into four VCGs, with one containing at least nine subgroups (48) displaying major differences in aggressiveness to potato (25,48,51). Viji et al. (52) studied the relationship among isolates of *Sclerotinia homoeocarpa* and found a correlation between VCG and amplified fragment length polymorphism (AFLP) group. They also observed a relationship between virulence on turf grass and VCG in which certain virulence groups corresponded to specific VCGs. Nitzan et al. (37) first reported four VCGs among 110 isolates of *C. coccodes* from Israel, France, and the Netherlands, demonstrating the presence of genetically diverse subgroups within that species. Recently, 123 North American isolates of *C. coccodes* were classified to six VCGs using the same technique (36). In both instances, results of pathogenicity studies indicate that differences in aggressiveness could be correlated with differences in vegetative compatibility.

Vegetative compatibility analysis is a laborious, time-consuming procedure that relies on the generation of nitrate nonutilizing (*nit*) mutants of each isolate, the phenotypic characterization of those mutants, and the ability of the mutant isolates to anastomose with selected tester isolates (24). This can be further complicated by the inability of some mutants to complement tester strains. In all, 27% of the European *C. coccodes* isolates previously evaluated for VCG and 18% of the North American isolates did not anastomose with selected VCG tester isolates and, therefore, could not be assigned to a VCG (36,37). Analysis of the population structure of *C. coccodes* would be enhanced by a more rapid and robust method of distinguishing genetically divergent isolates. Biochemical and molecular techniques including isozymes (7), polymerase chain reaction (PCR) (13), DNA sequencing (22,35,44,47), restriction fragment length polymorphism (RFLP) (11), random amplified polymorphic DNA (RAPD) (4,14,16,21,34), and AFLP (3,38,43) have been applied to a number of *Colletotrichum* spp. pathogenic to bean, strawberry, alfalfa, and potato. The use of these techniques made species identification less labor intensive and revealed significant genetic variation within the species. The use of DNA fingerprinting to distinguish among VCGs of *V. dahliae* has been demonstrated utilizing RFLP and RAPD techniques (19,20).

Characterization of *Colletotrichum* spp. using a variety of methods, traditional and molecular, has shown considerable amounts of variation, which often cause species to cluster into just a few groups (3,16,22,34–38,43,47,52,53). RAPD analysis by Xiao et al. (54) led to the separation of 52 *C. gloeosporioides* isolates into two clusters, each with considerable internal diversity. Using RAPD and ribosomal internal transcribed spacer (ITS) sequence analyses, Denoyes-Rothan et al. (16) and Martinez-Culebras et al. (35) likewise separated *C. acutatum* into two clusters. A strain-specific PCR marker derived from a RAPD band was reported by Dauch et al. (14) for a velvetleaf-derived strain of *C. coccodes*. Ansari et al. (3) utilized AFLP analysis to study diversity in 86 isolates of *C. lindemuthianum* representing 30 pathogenic races from 10 countries in Central and South America, Europe, and Africa. They concluded that the isolates could be grouped into three clusters which displayed some geographic association but no association with race classifications. In contrast, AFLP analysis revealed little variation among *C. coccodes* isolates from across the United Kingdom, possibly due to small sample size or limited geographic area (31).

DNA polymorphisms derived from AFLP analysis can be utilized as markers for populations, genotypes, phenotypes, and other characteristics. AFLP analysis has been used for differentiation of species of *Colletotrichum* pathogens of alfalfa (38) and the characterization of geographic variability among *C. lindemuthianum* isolates from Europe, South America, and Africa (3). *C. lindemuthianum* populations from Mexico were found to contain a large degree of genotypic variability within isolates taken from just a single plant (43). Diversity in several species has been evaluated comparing AFLP and VCG techniques. Strains of *Fusarium oxysporum* pathogenic or nonpathogenic on tomato separated into different clusters based on AFLP analysis (5). Although some bands did appear to show specificity for pathogenic or nonpathogenic strains, some nonpathogenic isolates clustered with the pathogenic group (5). AFLP analysis of the dry rot fungus *Serpula lacrymans* revealed a very low level of genetic diversity (29).

The initial objectives of this research included the use of AFLP to study relationships within and among North American VCGs. Although vegetative compatibility determination takes at least 10 to 12 weeks following isolate purification, AFLP analysis can be done in 2 weeks or less, significantly reducing the time and effort involved. This allowed its use as an efficient molecular technique that could facilitate timely, in-depth studies on the biology and ecology of *C. coccodes* populations affecting potato. Results with AFLP have allowed us to expand the original goal to include the rapid identification of different classes of *C. coccodes* which correspond to established VCGs and to use this technique to expand the number of isolates studied.

MATERIALS AND METHODS

Collection and storage of *C. coccodes* isolates. Isolates of *C. coccodes* were collected from infected plants in fields from potato-producing regions of the United States and Canada (Table 1). In all, 21 isolates were obtained from the culture collections of J. Miller, University of Idaho, H. Dillard, Cornell University, and R. Rowe, The Ohio State University. A small number of isolates received were collected from hosts other than potato, including six from tomato, one from red pepper, and three from peppermint. Researchers at North Dakota State University collected 98 isolates of *C. coccodes* isolated from tubers, stems, roots, and stolons of potato tissue surface sterilized in 10% bleach, rinsed thoroughly, dried, and plated onto Sorenson's NP-10 semi-selective media (28,46) prepared in two parts. Part A consisted of 5 g of polygalacturonic acid, sodium salt (PGA) (P3850; Sigma) in 500 ml of water. Part B consisted of 15.0 g of Bacto Agar, 1.0 g of KNO_3, 1.0 g of KH_2PO_4, 0.5 g of KCl, 0.5 g of $MgSO_4 \cdot 7H_3O$, and 0.5 ml of Niaproof (Tergitol) (N1404; Sigma) in 500 ml of distilled water. After autoclaving and cooling parts A and B separately, 50 mg each of filter-sterilized chloramphenicol and chlorotetracycline-HCl dissolved in methanol, as well as 50 mg of streptomycin sulfate dissolved in water, were added to part B. Parts A and B were mixed slowly and the media was immediately poured. Agar sections containing *C. coccodes* grown from tissue were transferred to water agar for monoconidial isolation. Isolates also were hyphal tipped if contamination persisted. The 92 *C. coccodes* isolates collected by Washington State University were subcultured to 1% potato dextrose agar (PDA) consisting of 10.0 g of PDA, 15.0 g of Bacto agar, and 0.1 g of streptomycin sulfate in 1 liter of distilled water, incubated in the dark at 25°C for 7 days. Monoconidial isolates were obtained by micromanipulation of conidia streaked to Czapek Dox agar (CDA) (37).

Homogeneous cultures of *C. coccodes* were grown on clarified V8 medium (CV8) (100 ml of Campbell's V8 juice, 1.5 g of $CaCO_3$, 15 g of agar, and 900 ml of water) amended with ampicillin at 50 µg/ml (CV8+Amp) for 5 to 7 days and were used to inoculate 100-ml liquid cultures of Richard's solution (55). Richard's solution was prepared with 10.0 g of sucrose, 10.0 g of KNO_3, 5.0 g of KH_2PO_4, 2.5 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $FeCl_3 \cdot 6H_2O$, and 150 ml of V8 juice in 1 liter of distilled water. Liquid cultures were agitated at 25 ± 2°C for 12 to 14 days. After 8 to 10 days, a 100-µl aliquot was aseptically transferred to CV8 agar and incubated at 25 ± 2°C for 5 to 7 days to ensure that isolate integrity had been maintained. Permanent stock cultures were established on silica gel crystals stored at –80°C using microsclerotia scraped

from a CV8+Amp agar plate in a 7.5% skim milk solution (45). All further experiments were conducted using the isolates obtained from hyphal tipping or single conidial purification.

VCG analysis. Vegetative compatibility was characterized with *nit* mutants using methods previously described (9,12,30,36). To select *nit* mutants, agar pieces with fungal mycelia of each isolate were placed on water agar chlorate medium (WAC) as described by Korolev and Katen (30). Colonies with thin white or green transparent mycelia were considered *nit* mutants and a single conidium was transferred to CDA and incubated for 7 days at 25°C in the dark. Complete phenotyping and separation of *Nit1* from *nit3* and *NitM* from *nit2* is unnecessary for VCG analysis and, therefore, was not performed (37). Partial phenotyping of the *nit* mutants was done only to distinguish *nit1*/*nit3* mutants from *NitM*/*nit2* mutants. Two mycelia plugs of each isolate were placed on both CDA and CDA amended with 0.02% hypoxanthine and incubated at 25°C in the dark for 5 days. Colonies with wild-type growth on CDA amended with hypoxanthine and a thin mycelium on CDA were considered *nit1*/*nit3* mutants, whereas colonies with a thin mycelium on both media were considered *NitM*/*nit2* mutants.

Complementation between *nit* mutants was examined on CDA containing nitrate as the sole nitrogen source. Mycelia plugs (1 by 1 mm) of *NitM*/*nit2* and *nit1*/*nit3* mutants were placed 1 cm apart in a 5-cm petri plate. Plates were incubated for 14 days at 25°C in the dark. Complementation was characterized by the formation of a heterokaryon with wild-type growth at the contact zone of the two mutants. *NitM*/*nit2* mutants that demonstrated the ability to anastomose with a large number of other mutants were chosen as VCG tester strains. All testers demonstrated an ability to form stable heterokaryons only with isolates from their own VCG. VCGs were defined as the isolates anastomosing with given sets of tester strains. Eight isolates were selected as VCG testers, representing six VCGs. In addition to the VCG tester strains selected from the North American population, isolates also were examined in complementation experiments using tester strains selected from a European population (37).

Pathogenicity trials. Seven isolates, three originating from potato and four originating from non-potato crops, were selected for pathogenicity trials. The trials were conducted in a greenhouse on the Pullman campus of Washington State University during 2003 and were repeated during 2004 with the same set of isolates. Temperatures during the trials were 18 ± 4.4 and 18 ± 4.9°C in 2003 and 2004, respectively. The experimental design was a randomized complete block with seven replications per isolate and seven noninoculated control plants.

Plant material. Disease-free, nuclear potato seed tubers of the *C. coccodes*-susceptible cv. Russet Norkotah were greenhouse produced in Montana by a commercial seed potato grower. Tubers were washed with soap and warm water to remove excess soil deposits, surface sterilized (1% NaClO for 10 min), allowed to dry at ambient temperature, and cut into seed pieces (averaging 24 g). Seed pieces were allowed to partially suberize for 48 h at 10°C prior to planting.

Preparation of inoculum. Fungal inoculum for soil application was prepared on rye seed in 1-liter flasks. Rye seed were washed with distilled water, transferred to flasks (1/3 of flask volume), and autoclaved twice (45 min at 120°C) with a 24-h interval (25°C in the dark) between each autoclave cycle. Isolates were cultured on 1% PDA, as described above, in 9-cm petri plates for 7 days at 25°C in the dark. Fungal growth from one petri plate was transferred in 1-cm^2 sections into each flask. Flasks were capped with autoclaved cotton wool and aluminum foil, shaken well, and incubated at 25°C in the dark until rye seed were fully colonized by the fungus (3 to 4 weeks). Rye seed were allowed to air dry at room temperature (14 days at 23°C), and maintained at room temperature in sealed plastic bags. After dehydration, a randomized sample of 10 rye seed was taken from each flask. The number of sclerotia on a single, fully colonized rye seed was counted using a dissecting microscope, and ranged from 150 to 200.

Soil inoculation. Soil inoculation was performed by incorporating 15 g of rye seed infested with *C. coccodes* into 11-liter pots containing potting mixture (Sun-Shine LC-1; Sun-Gro Horticulture Distributor Inc.) and inoculum was mixed by hand to an even distribution. Uninfested, autoclaved rye seed served as control. One seed-tuber piece was planted in the middle of each pot ≈1 cm below the surface. Pots were irrigated immediately after planting, and were not irrigated again until plant emergence.

Cultural practices. Plants were irrigated every 2 days after emergence. Osmocote (12 g; 16-16-16, N-P-K) was applied once, before application of inoculum and planting of seed-tuber pieces, and thoroughly mixed into the soil. Two months after planting (29 April 2003 and 23 April 2004), plants were pruned to one stem per plant in order to standardize the number of stems per plant among all replications.

Evaluation of pathogenicity. Pathogenicity was evaluated after plants died. Stems were thoroughly air dried in pots on the

TABLE 1. Origin, vegetative compatibility group (VCG), and amplified fragment length polymorphism (AFLP) designation for 112 and 211 *Colletotrichum coccodes* isolates, respectively

Location	NA-VCG1/ AFLP1	NA-VCG2/ AFLP2	NA-VCG3/ AFLP3	NA-VCG4/ AFLP4	NA-VCG5/ AFLP5	NA VCG6/ AFLP6	Total	VCG not assigned
Alberta	0/0	5/5	1/2	0/0	0/0	0/0	6/7	1
British Columbia	0/0	0/0	1/1	0/0	0/0	0/0	1/1	0
Colorado	0/0	0/6	0/0	0/0	0/1	0/0	0/7	0
Idaho	1/4	4/10	1/1	0/0	2/7	0/0	8/22	2
Michigan	0/0	0/5	0/0	0/0	0/1	0/0	0/6	0
Minnesota	0/2	1/4	1/1	0/0	1/13	0/0	3/20	2
Montana	3/3	3/4	1/1	0/0	5/10	0/0	12/18	6
Nebraska	1/2	1/6	2/2	1/1	1/7	0/0	6/18	3
Nevada	0/4	0/11	0/1	0/0	0/2	0/0	0/18	0
New York	0/0	1/1	0/0	0/0	0/5	0/0	1/6	2
North Dakota	5/5	3/8	1/1	0/0	0/0	0/0	9/14	1
Ohio	0/0	0/0	0/0	0/0	0/0	2/2	2/2	0
Oregon	0/3	4/13	0/2	1/1	2/10	0/0	7/29	2
Pennsylvania	0/0	1/2	0/0	0/0	0/0	0/0	1/2	1
Saskatchewan	1/1	0/0	0/0	0/0	0/0	0/0	1/1	0
Texas	0/0	0/0	0/0	0/0	1/1	0/0	1/1	0
Washington	8/8	14/14	1/2	0/0	2/4	0/0	25/28	3
Wisconsin	0/0	0/0	0/0	0/0	2/3	0/0	2/3	1
Unknown	1/1	1/3	0/0	0/0	1/4	0/0	3/8	0
Total	20/33	38/92	9/14	2/2	17/68	2/2	88/211	24

greenhouse benches and analyzed using the following parameters. (i) Progeny tubers total yield weight (in grams) was calculated as percent yield reduction in relation to noninoculated control plants. (ii) Sclerotia density on roots (5 cm of stem below the soil level) and on crowns (5 cm of stem above the soil level) was visually evaluated on a scale of 0 to 5, where 0 = no sclerotia, 1 = 1 to 10%, 2 = 10 to 30%, 3 = 30 to 50%, 4 = 50 to 75%, and 5 = 75 to 100% of the surface area covered with sclerotia. (iii) Expansion of sclerotia on the aboveground portion of the stem (in centimeters) was determined by measuring the height to which sclerotia formed on the stem. (iv) Disease incidence on daughter tubers was determined by cutting a sample of vascular tissue from the stem end of 5 progeny tubers (total of 36 tubers per isolate), placing it onto 1% PDA, and incubating at 25°C in the dark for 7 days. Infection frequency was calculated by counting the number of tissues from which *C. coccodes* was recovered.

Extraction of genomic DNA. Pure cultures of *C. coccodes* grown in Richard's solution were filtered through Whatman no. 1 filter paper, frozen at –80°C, lyophilized, and ground to a powder in liquid nitrogen. DNA extraction was performed on 20 mg of the powder using the Qiagen DNeasy Plant Mini DNA kit (69104; Qiagen, Inc., Valencia, CA). DNA was eluted in a final volume of 200 µl in Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and assessed for size and purity by electrophoresis on a 1% agarose gel. To ensure species identity, PCR was performed on each isolate using the *C. coccodes*-specific primers Cc1NF1 and Cc2nR1 (13).

AFLP analysis. AFLP reactions were performed by a modification (1,58) of the method of Vos et al. (53) utilizing the Invitrogen AFLP Core Reagent kit (Invitrogen Corp., Carlsbad, CA). Genomic DNA (≈200 to 400 ng) was digested with *Eco*RI and *Mse*I. The 16-bp adapter-primer sequences supplied with the kit were ligated to the fragments at 20°C for 12 h. The reaction product was diluted 1:10 with TE buffer and the preamplification step was performed with primers complimentary to the adapter sequences plus a one-base selective 3′ extension (Table 2). Amplification was performed in a 25-µl volume using a PTC-200 programmable thermocycler (MJ Research, Waltham, MA). The reaction mix contained 200 µM dNTPs, 1× PCR reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 0.1% Triton X100), 36 ng of each primer, 2.0 µl of the diluted adapter-primer DNA, and 1 unit of *Taq* DNA Polymerase (Promega Corp., Madison, WI). The PCR reaction was programmed for 20 cycles of 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s. Each reaction mix was diluted 1:10 with TE buffer before use with the selective amplification reaction.

Selective PCR amplification was performed using primer sets with two-base extensions in addition to reaction components as described previously for the preamplification. The PCR parameters included an initial cycle of 30 s at 94°C, 30 s at 65°C, and 1 min at 72°C, followed by 12 cycles with stepwise lowering of the annealing temperature by 0.7°C in each cycle (touchdown profile) and 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The resulting selectively amplified PCR products were analyzed by electrophoresis on a 5% acrylamide/7 M urea denaturing gel in 0.5× Tris-borate EDTA at a constant power of 65 W for 3 h and stained using the Silver Sequence DNA Sequencing System (Q4132; Promega Corp.) (56). Selective amplifications initially were carried out with 10 combinations of primer pairs with two-base selective extensions: E-AC/M-CC, E-AC/M-CA, E-AC/M-CG, E-AC/M-CT, E-AT/M-CC, E-AT/M-CT, E-AT/M-CG, E-AG/M-CC, E-AG/M-CA, and E-AG/M-CG. Based on the number of polymorphisms generated, E-AC/M-CC, E-AG/M-CC, and E-AT/M-CC subsequently were selected to screen the *C. coccodes* collection (Table 2). The isolates of *C. coccodes* clustered by VCG were analyzed by AFLP analysis in a blind study in which VCG was not known prior to being analyzed by AFLP.

TABLE 2. Oligonucleotides utilized in amplified fragment length polymorphism selective amplification polymerase chain reaction for classification of *Colletotrichum coccodes* isolates into a corresponding vegetative compatibility group

Primer	
Forward primers	
Eco-AC	5′ GACTGCGTACCAATTCAC 3′
Eco-AG	5′ GACTGCGTACCAATTCAG 3′
Eco-AT	5′ GACTGCGTACCAATTCAT 3′
Reverse primer	
Mse-CC	5′ GATGAGTCCTGAGTAACC 3′

Data analysis. Individual bands between 150 and 600 bp were scored as present or absent. For each of the *C. coccodes* isolates, binomial data from the three selective amplification reactions was combined and used to create a similarity matrix using the WinDist application of the WinBoot program (International Rice Research Institute, Manila, Philippines) and the Dice similarity coefficient. The sequential, agglomerative, hierarchical, nested clustering method (SAHN) module of the numerical taxonomy and multivariate analysis system (NTSYS) (pc 2.1 program; Exeter Software, E. Setauket, NY) was used to do unweighted pairgroup method with arithmetic means (UPGMA)-based cluster analysis. The strength of these relationships was estimated by bootstrap analysis of 1,000 reiterations performed using the SEQBOOT tool of the Phylogeny Inference Package (PHYLIP, University of Washington). Consensus phylogenic trees were created using the CONSENSE tool of PHYLIP. VCGs determined by traditional methods and clusters derived from AFLP were correlated (PROC CORR) utilizing SAS (version 9.1; SAS Institute; Cary, NC). Single degree-of-freedom comparisons (α = 0.05) were performed on pathogenicity parameters with plant host (potato versus non-potato) as main fixed effect utilizing the general linear model procedure (PROC GLM) in SAS.

The ability of the AFLP data to identify VCGs was tested using principal components analysis (PCA) to reduce the dimensionality of the AFLP data set (42), followed by discriminant analysis to predict VCG group membership (23). In the process of using the multivariate PCA technique, the original variables, in this case the AFLP bands, were transformed into a set of new variables or principle components (PCs) which represent linear combinations of the original data. One of the useful properties of the PCs is a lack of correlation, because they are orthogonal and "sorted" according to the amount of variation they explain. The first PC is the linear combination of the original variables that accounts for the greatest amount of variation, the second is the linear combination that accounts for the next greatest amount of variation, and so on. If the original variables show strong correlations among some of the variables, chances are good that a small number of PCs will account for a large proportion of the variation in the data set. If the original variables are largely uncorrelated, then PCA will not likely have much effect. Two general principles often have been used to decide how many PCs to retain in a data set: (i) PCs with eigenvalues greater than one and (ii) PCs up to and including the one in which cumulative variation described is ≥90%.

Two discriminant analysis models were used, linear discriminant analysis (LDA) and quadratic discriminant analysis (QDA), in an attempt to classify *C. coccodes* into presumptive VCGs. Initially, these models were based upon only the first 13 PCs, which accounted for a large percentage (>85%) of the total variation among the original variables (Table 3). Error rates were assessed after each model fit. In an attempt to achieve a more parsimonious model, the least important PCs were deleted sequentially and error rates were evaluated after each run. Using this methodology, a quadratic discriminant model was identified which required only the first three PCs to achieve a very high

VCG classification accuracy (>90%) based solely on AFLP analysis. In all, 88 isolates were available for the PCA and discriminant analysis with VCG and 87 bands from the AFLP analysis. Several bands were dropped because they were identical for all 88 isolates. The previously determined VCG and presumptive VCG as determined by AFLP were correlated (PROC CORR) using SAS. The difference in frequency between VCGs and AFLP clusters among the six VCGs plus the nonassigned isolates were compared using the χ^2 test.

RESULTS

VCG analysis. Vegetative compatibility analysis utilizing *nit* mutants was performed on 112 *C. coccodes* isolates, resulting in the assignment of 88 isolates into six VCGs, designated NA-VCG 1, 2, 3, 4, 5, and 6 (Table 1). Among the six VCGs, 38 isolates (34%) were assigned to NA-VCG2, 20 (18%) were assigned to NA-VCG1, 17 (15%) were assigned to NA-VCG5, and 9 (8%) were assigned to NA-VCG3. Two (2%) isolates each were assigned to NA-VCG4 and NA-VCG6. Twenty-four isolates (22%) did not anastomose with any tester isolate, could not be assigned to any VCG, and, therefore, were designated as "non-assigned." Only two of these isolates were self-compatible. No complementation was detected between North American isolates examined and any of the European VCG tester strains.

AFLP analysis. In all, 211 *C. coccodes* isolates, including the 112 analyzed by VCG analysis (37), were evaluated by AFLP utilizing three selective primer sets (Table 2). In total, 90 bands between 150 and 600 bp were scored as present or absent for each isolate. Several polymorphic bands could be used to presumptively distinguish VCGs in most cases, although complete differentiation of VCGs required computer analysis of the entire banding pattern produced by all three primer sets (Fig. 1). *C. coccodes* isolates belonging to NA-VCG1 could be distinguished presumptively by the presence of bands at 340 and 170 bp with primer sets E-AC/M-CC and E-AG/M-CC, respectively, whereas isolates belonging to NA-VCG3 could be distinguished by a band at 420 bp with primer set E-AC/M-CC. A band at 235 bp in the E-AG/MCC-amplified samples and another at 275 bp in the E-AT/M-CC-amplified samples were present in all isolates belonging to NA-VCG5 and a single NA-VCG4 isolate. NA-VCG1 and NA-VCG3 isolates displayed bands at 220 bp in E-AC/M-CC-amplified samples and 410 bp in E-AT/M-CC-amplified samples, whereas bands at 175 bp were present in NA-VCG2 and NA-VCG5 with E-AT/M-CC amplification. The *C. coccodes* isolate belonging to NA-VCG6 displayed numerous unique polymorphic bands and could be distinguished easily from the other NA-VCGs visually.

UPGMA-based cluster analysis of the gel banding patterns separated the *C. coccodes* isolates which were analyzed for VCG into five distinct clusters (Fig. 2). Reproducibility of the bootstrap analysis of these five major groups was between 54 and 100%. Isolates of *C. coccodes* belonging to NA-VCG1 and NA-VCG3 displayed closely related patterns, as did isolates belonging to NA-VCG2 and NA-VCG5. Bootstrap analysis of the divergence of these groups resulted in NA-VCG2 and NA-VCG5 having divergence in only 67% of the 1,000 reiterations, whereas the NA-VCG1 and NA-VCG3 isolates were divergent in 82% of the reiterations (Fig. 2). However, the branches of the dendrogram containing the NA-VCG2/NA-VCG5 group and the NA-VCG1/NA-VCG3 group separated in 99% of the reiterations (Fig. 2). The two *C. coccodes* isolates belonging to NA-VCG6 were the most divergent from other NA-VCGs. These clusters or branches closely matched the previously determined NA-VCGs. Of the 88 isolates with a designated VCG, 82 (93%) clustered with others of the same VCG resulting in a strong (r = 0.932) and highly significant ($P < 0.0001$) correlation. All 20 *C. coccodes* isolates belonging to NA-VCG1, 35 of 38 isolates belong to NA-VCG2, 8 of 9 isolates belonging to NA-VCG3, 17 of 17 isolates belonging to NA-VCG5, and both VCG6 isolates clustered together. The two isolates belonging to NA-VCG4 did not cluster together. One clustered with isolates belonging to NA-VCG2 while the other clustered with isolates belonging to NA-VCG5. There were no distinct branches for any of the nonassigned isolates. Among these nonassigned isolates, one clustered with isolates belonging to NA-VCG1, nine with isolates belonging to NA-VCG2, nine with isolates belonging to NA-VCG5, and five with the two NA-VCG6 isolates.

An additional 99 isolates of *C. coccodes* were evaluated by AFLP but had no previous VCG determination. The *C. coccodes* isolates analyzed only using AFLP clustered in the same five branches as the original 112 *C. coccodes* isolates with designated NA-VCGs (Table 1; Fig. 3). AFLP analysis of all 211 isolates clustered 35 isolates in NA-VCG1, 93 in NA-VCG2, 12 in NA-VCG3, 46 in NA-VCG5, and 25 in NA-VCG6. Within the NA-VCG6 branch, non-potato isolates clustered together on a distinct sub-branch, separate from 15 of the 17 potato isolates in this group. Of 10 total isolates from non-potato hosts, 8 clustered with isolates of NA-VCG6 (5 from tomato, 2 from red pepper, and 1 from peppermint), potentially indicating some genetic differentiation between the potato and non-potato isolates. Two non-potato isolates of *C. coccodes,* both from peppermint, clustered with isolates from NA-VCG2 and NA-VCG5.

LDA, using equal priors and 13 PCs as predictors, yielded a total misclassification rate of 6 of 88 (6.8%) of *C. coccodes* isolates for which VCG data existed. QDA on the same data resulted in only one isolate from VCG2 being misclassified into VCG1, an error rate of approximately 1.1%. When each model was reduced to using the first three PCs, the LDA error rate remained 6 of 88 (6.8%), whereas the QDA error rate increased to 4 of 88 (4.5%). In the QDA model, three isolates from VCG2 were misclassified (one each in VCG1, VCG3, and VCG5) and one isolate from VCG3 was misclassified into VCG2. The LDA model makes these same errors as well as misclassifying the two VCG4 isolates, one each into VCG2 and VCG5. The first three PCs (those used in the final discriminant analysis models) accounted for just over 60% of the variation in the original data set (Table 3).

Using a plot of the third PC versus the first PC, nearly all of the VCGs separated readily into clusters using only these two composite variables, indicating that it is possible to correctly classify

TABLE 3. First 15 principal components (PCs) and the proportion of variation in the 87 original binary variables accounted for by each[w]

PC	Eigenvalue[x]	Proportion[y]	Cumulative[z]
1	23.73	0.27	0.27
2	16.42	0.19	0.46
3	12.63	0.15	0.61
4	7.54	0.09	0.69
5	4.82	0.06	0.75
6	2.70	0.03	0.78
7	2.00	0.02	0.80
8	1.80	0.02	0.82
9	1.77	0.02	0.84
10	1.33	0.02	0.86
11	1.19	0.01	0.87
12	1.16	0.01	0.89
13	1.09	0.01	0.90
14	0.90	0.01	0.91
15	0.81	0.01	0.92

[w] The percentage of variation explained by each individual PC is determined by multiplying the proportion values by 100.
[x] Eigenvalue is the variance of each PC.
[y] Proportion represents the proportion of the total variance explained in a set of PCs.
[z] Cumulative is the running sum of the proportion of the variance explained by the PCs.

most of the isolates based upon only these two dimensions (data not shown). Through the use of the QDA model, which does not assume equal variances across groups like LDA, and with the addition of PC2, we are able to accurately predict the VCG of 84 of the 88 (95%) of the *C. coccodes* isolates using AFLP analysis. The correlation between these two methods was strong (r = 0.970) and highly significant ($P < 0.0001$).

A presumptive VCG assignment was determined for all 99 isolates of *C. coccodes* which were evaluated by AFLP but not VCG using the QDA model. VCG determinations by this model

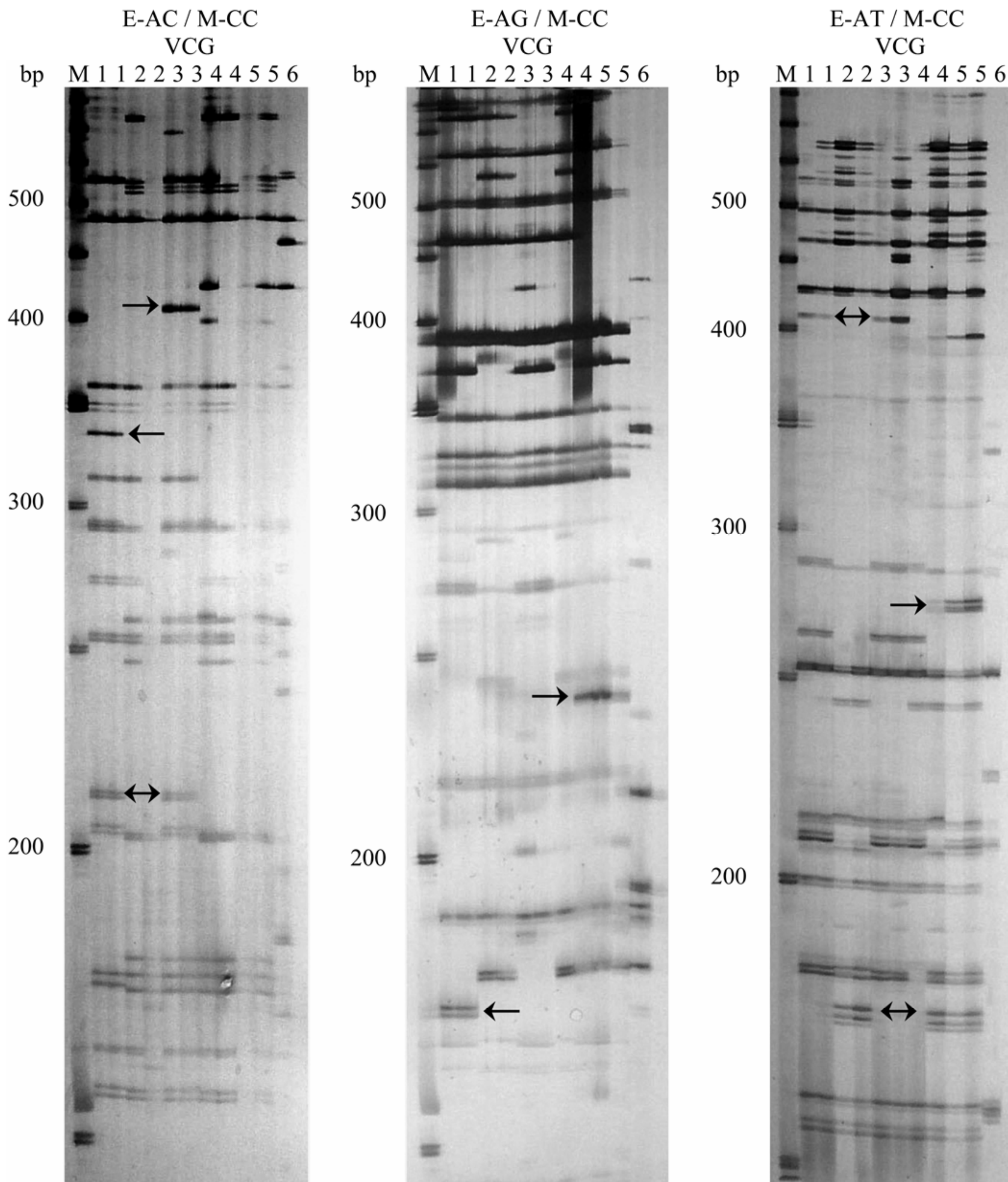

Fig. 1. Amplified fragment length polymorphism electrophoresis of *Colletotrichum coccodes* isolates from each vegetative compatibility group (VCG). Selective polymerase chain reaction (PCR) amplification was performed utilizing primer sets E-AC/M-CC, E-AG/M-CC, and E-AT/M-CC with 2-bp extensions. PCR products were separated by electrophoresis in a 5% polyacrylamide/7 M urea gel followed by silver staining. Polymorphic bands used to distinguish VCGs are indicated by arrows. A standard size markers of 50-bp intervals is denoted as lane M.

closely matched those made using the cluster analysis, with the exception of VCG6. All isolates which clustered with those of VCG6 were classified as VCG5 by the model. The frequency distribution among the AFLP groups, including all 211 isolates, was significantly different from the frequency distribution of the initial 112 isolates whose VCG was determined by traditional methods (χ^2 =56.25, df = 6, $P < 0.0001$) (Fig. 4). This significant difference can be attributed mainly to the additional 51 *C. coccodes* isolates classified as NA-VCG5 and the 24 isolates which could not be assigned to a VCG by traditional methods (Fig. 4).

Overall, little geographic pattern was observed among VCG or AFLP groups (Table 1). Of the 19 states and provinces represented, 13 had isolates from more than one VCG. *C. coccodes* isolates belonging to NA-VCG2 were found in 13 states from New York to Washington and other groups were similarly widespread. Several VCGs often were recovered from the same area or field (data not shown). Isolates belonging to all groups distinguished by AFLP, except group 4, were found in Washington State, and three VCGs were represented among samples collected near Hermiston, Oregon.

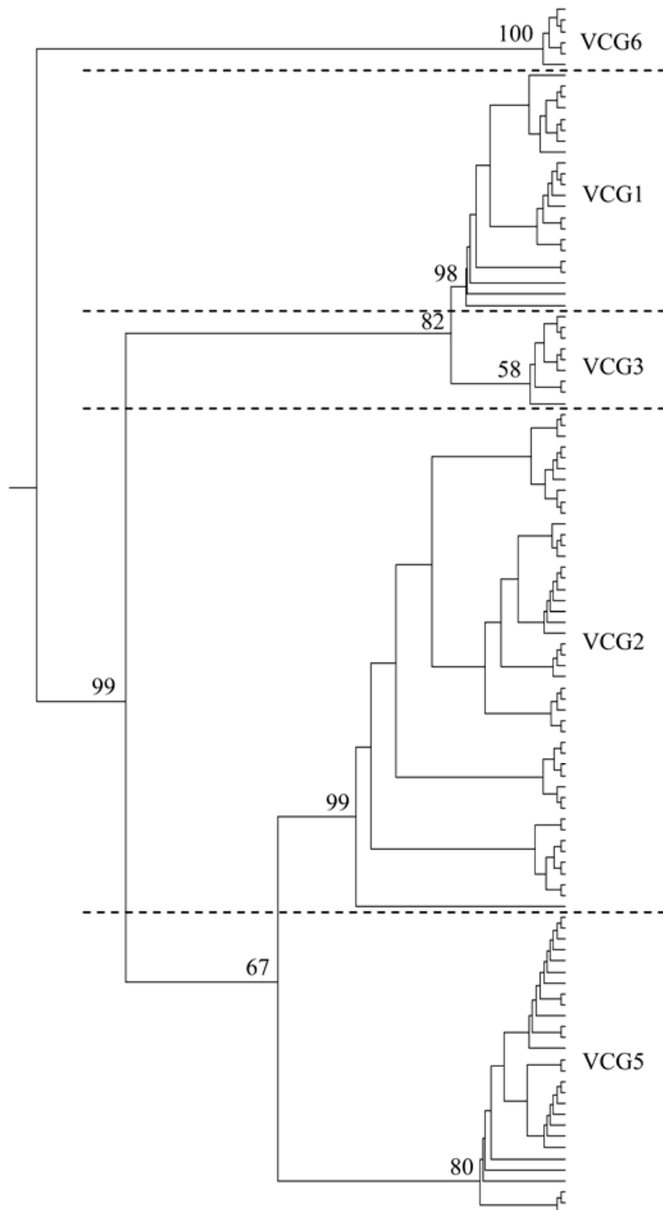

Fig. 2. Consensus dendrogram generated by 1,000 bootstrap reiterations using the SEQBOOT and CONSENSE applications of Phylogeny Inference Package (PHYLIP) to analyze amplified fragment length polymorphism (AFLP) data of 112 *Colletotrichum coccodes* isolates for which the vegetative compatibility group (VCG) had been determined by traditional methods. The dendrograms were generated with unweighted pair group method with arithmetic cluster analysis performed with the sequential, agglomerative, hierarchical, nested clustering method module of Numerical Taxonomy and Multivariate Analysis System pc2.1 on a similarity matrix of 90 AFLP bands that were scored as present or absent. The numbers at the major nodes indicate the percentage of times the group of isolates at the left of the node occurred among the 1,000 bootstrap-generated dendrograms. The dotted horizontal lines designate branches of the dendrogram corresponding to the VCG to which the isolates were assigned, indicated on the right.

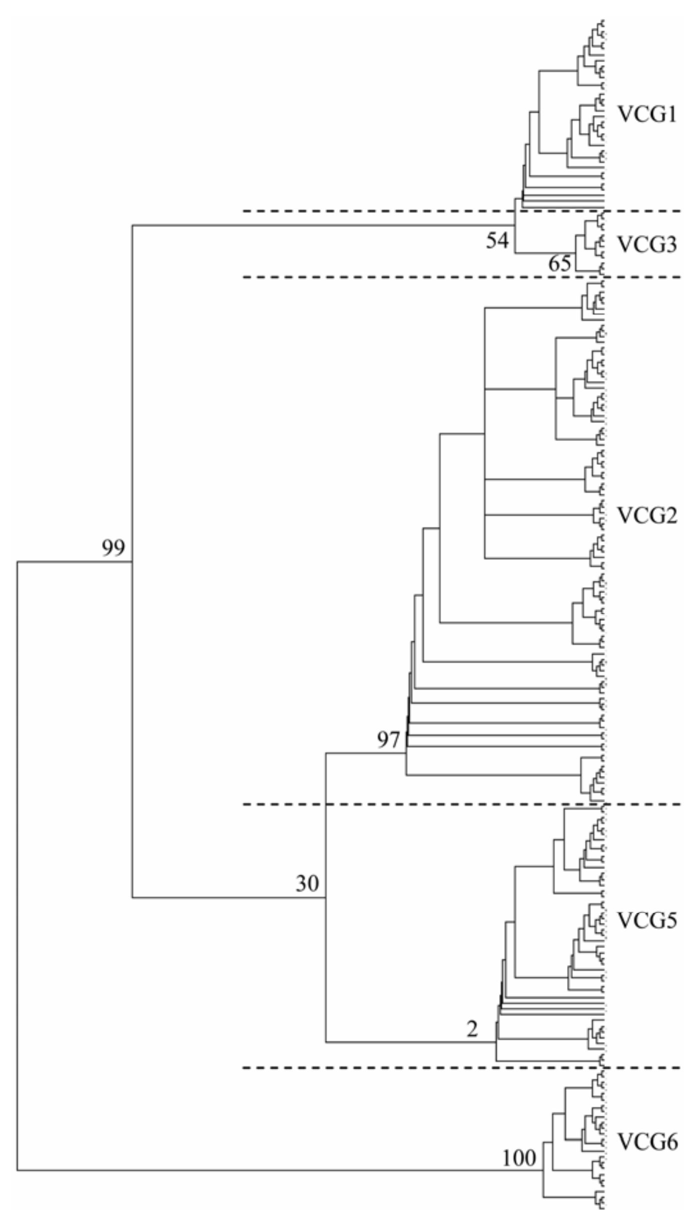

Fig. 3. Consensus dendrogram generated by 1,000 bootstrap reiterations using the SEQBOOT and CONSENSE applications of Phylogeny Inference Package (PHYLIP) to analyze amplified fragment length polymorphism (AFLP) data of 211 *Colletotrichum coccodes* isolates, 112 of which previously had been characterized for vegetative compatibility group (VCG). The dendrograms were generated with unweighted pair group method with arithmetic cluster analysis performed with the sequential, agglomerative, hierarchical, nested clustering method module of Numerical Taxonomy and Multivariate Analysis System pc2.1 on a similarity matrix of 90 AFLP bands that were scored as present or absent. The numbers at the major nodes indicate the percentage of times the group of isolates at the left of the node occurred among the 1,000 bootstrap-generated dendrograms. The dotted horizontal lines designate branches of the dendrogram corresponding to the VCG to which the isolates were assigned, indicated on the right.

As part of the collection process, data were kept on the potato tissues from which the isolates were derived. Isolation of *C. coccodes* was attempted from all tissue with visible black dot symptoms. In all, 167 isolates were documented as originating from potato stems, roots, stolons, and tubers, with the majority of isolates recovered from tubers ($n = 91$) and stems ($n = 65$) (Table 4). In some instances, this information was not available for isolates received from cooperators. There appeared to be no specificity for either tubers or stems among any VCGs.

Pathogenicity trials. Seven isolates of *C. coccodes*, three collected from potato hosts, and four collected from non-potato hosts were examined for pathogenicity on potato. Among the three potato isolates, two were classified to NA-VCG4 and one was nonassigned by traditional VCG methods. Among the four isolates collected from non-potato hosts, two originated from tomato and red pepper and were classified as NA-VCG6, and two originated from tomato and mint and were nonassigned by traditional methods. However, the AFLP analysis classified them to NA-VCG5. Potato plants inoculated with *C. coccodes* isolates originating from both potato and non-potato displayed disease symptoms on stems and daughter tubers.

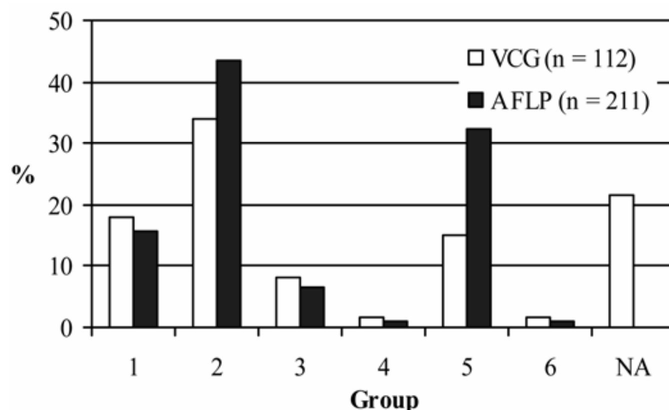

Fig. 4. Frequency distribution of percentage of 112 *Colletotrichum coccodes* isolates assigned to vegetative compatibility groups (VCGs) by traditional methods versus 211 *C. coccodes* isolates designated using a quadratic discriminant analysis model of amplified fragment length polymorphisms (AFLP). NA denotes isolates not assigned to any VCG.

Sclerotial density and sclerotial expansion were greater in plants infected with *C. coccodes* isolates originating from potato than on plants inoculated with isolates originating from non-potato hosts in 2003, but not in 2004 (Table 5). Percentage of infected progeny tubers was greater in plants infected with potato isolates than in plants infected with non-potato isolates in 2004, but not in 2003 ($P = 0.09$) (Table 5). Yield did not statistically differ both years, either between plants inoculated with potato or non-potato isolates or in relation to the control (Table 5).

DISCUSSION

Results for *C. coccodes* from this research are consistent with results from other *Colletotrichum* spp., indicating considerable genetic diversity within the species but a diversity that is compartmentalized into a limited number of groups. For the first time with *C. coccodes,* we have combined the molecular technique of AFLP with vegetative compatibility grouping to analyze the amount of variation among North American isolates and determine whether these two methods yield comparable results. A remarkable degree of congruence between the results from the two techniques was found. VCG and AFLP analysis cluster *C. coccodes* isolates into the same groups in the majority (82 of 88) of isolates examined. Similarly, a close association between the VCG and the AFLP haplotype was determined by Zeller et al. (57) with *Gibberella zea* and Viji et al. (52) with *Sclerotinia homoeocarpa*. In contrast, a lack of correlation was found with the dry rot fungus *Serpula lacrymans*. In that case, AFLP polymorphisms were independent of VCG, possibly due to the presence of a sexual cycle in this pathogen (29). We believe that these studies report, for the first time, a relationship of specific AFLP bands to corresponding VCGs of *C. coccodes*. These bands also appear in the isolates that could not be assigned to a VCG; therefore, it is possible they could be indicators of further diversity within the groups similar to the subgroups of *V. dahliae*. Diversity studies with *G. zeae* also indicated that VCG and AFLP identified the same subsets of isolates (56) but did not specify any identifying bands.

AFLP patterns from all 211 of the North American *C. coccodes* isolates separated into five groups, coinciding almost completely

TABLE 4. Plant tissue origin of 167 *Colletotrichum coccodes* isolates from potato evaluated for presumptive vegetative compatibility group utilizing amplified fragment length polymorphism (AFLP) analysis

Plant tissue	AFLP1	AFLP2	AFLP3	AFLP4	AFLP5	Total
Stem	12	29	4	0	20	65
Tuber	18	39	9	2	23	91
Root	1	3	1	0	2	7
Stolon	1	1	0	0	2	4

TABLE 5. Pathogenicity parameters of seven isolates of *Colletotrichum coccodes* collected from potato and non-potato host plants[u]

		Sclerotial density (0–5)[v]		Sclerotial expansion (cm)[w]		Infected progeny tubers (%)[x]		Yield (g)	
Plant host[y]	No. tested[z]	2003	2004	2003	2004	2003	2004	2003	2004
Potato	3	3.8 a	2.9	11.0 a	10.5	14.7	21.0 a	515	429
Non-potato	4	3.1 b	3.3	9.4 b	7.6	6.2	3.7 b	474	460
Control	…	0	0	0	0	0	0	550	453

[u] Single degree of freedom comparisons ($\alpha = 0.05$) for plant host (potato versus non-potato) as main fixed effect were conducted using the general linear model procedure (Proc GLM) in SAS. Noninoculated control plants were included only in the statistical analysis of yield weight.

[v] Sclerotial distribution on root and crown in a 0-to-5 scale, where 0 = no sclerotia, 1 = 1 to 10%, 2 = 10 to 30%, 3 = 30 to 50%, 4 = 50 to 75%, and 5 = 75 to 100% of the surface area covered with sclerotia. Letters within columns denote significant differences.

[w] Expansion of sclerotia (height of spread) in aboveground part of stem measured.

[x] Disease incidence on progeny tubers—a sample of the vascular tissue from the stem end of five progeny tubers from each isolate repetition (total of 35 tubers per isolate) was cut and placed onto 1% potato dextrose agar. The percentage of infected tubers was evaluated.

[y] Plant host: three isolates from potato (two NA-VCG4 and one nonassigned vegetative compatibility group [VCG]), two isolates from tomato (1 NA-VCG6 and 1 nonassigned VCG [two NA-AFLP5]), one isolate from red pepper (NA-VCG6), and one isolate from peppermint (nonassigned VCG [NA-AFLP5]).

[z] Number of tested isolates.

with the six VCGs of Nitzan (36). The strong correlation between results of the two methods was sufficient to assign a presumptive VCG to 99 isolates not analyzed for vegetative compatibility and also for those isolates that did not react with NA-VCG tester isolates. The fact that there was little change in frequencies of the NA-VCG groups, with the exception of NA-VCG6, after the addition of the AFLP samples (Fig. 4) provides evidence that the predictions are accurate. The existence of isolates which do not anastomose with selected tester isolates could be explained by subgroups within larger VCGs, as has been demonstrated in *V. dahliae* (24,46).

Clearly, these two very different methods, although not measuring the same parameters, are detecting the same basic level of genetic variation. AFLP is a measure of overall DNA polymorphism among the organisms, whereas VCG is a phenotype that results from variation at one or a few genes. When VCG data are added to the dendrogram developed from AFLP data (Figs. 2 and 3), it is apparent that the VCG differences correspond to the major branches of the tree. There is considerable variation among the isolates within these large branches, but overall vegetative compatibility is consistent with the AFLP pattern. A possible explanation is that a rare genetic change in vegetative compatibility has isolated specific groups that have, over time, genetically diverged as a group. Occasionally, a mutation occurs and changes the vegetative compatibility again to another of a limited set of options, creating an isolate with the same AFLP pattern but a different VCG, as observed with the NA-VCG4 group. This rare group has no distinctive AFLP pattern and its two representatives have NA-VCG2 and NA-VCG5 AFLP patterns. This result possibly may represent a recent genetic change to the VCG genotype of two isolates that still retain the overall genotype of the parental isolates. The change could allow for anastomosis with other isolates of the new VCG and gene flow between genetically diverged isolates if they come into contact. This would be a rare event; however, dispersal of isolates with seed tubers would make it more probable. The lack of isolates with intermediate genotypes indicates that it is still a rare event. Isolates from the same areas with multiple VCGs also demonstrate the genetic isolation of distinct VCGs within the total population.

The lack of a geographic pattern for the dispersal of most VCGs indicates that, wherever genotypes and VCGs originated, they are now thoroughly dispersed throughout North American potato-growing regions, often more than one in the same field or sample. Isolates with identical AFLP patterns also often were found in locations distant from one another. Given the relatively high incidence of *C. coccodes* on certified potato seed tubers (27), these results are not surprising. With the possible exception of the NA-VCG6 cluster, the VCGs and the AFLP genotypic groups were scattered and intermixed. Geographic clustering of potato isolates of NA-VCG6 in two areas of Nebraska and Minnesota may indicate a recent introduction of this isolate into those areas. The similarity of the NA-VCG6 potato isolates to isolates from non-potato hosts indicates a possible source. A majority of non-potato isolates were classified as NA-VCG6, divergent from most potato isolates; however, they were pathogenic to potato, although they did not cause a significant yield loss. More work comparing potato and non-potato isolates of *C. coccodes* needs to be performed to understand their ability to infect multiple crops and whether vegetative compatibility plays a role in this genetic variability.

Among AFLP groups, the QDA model and the cluster analysis classified 88% of the 211 isolates into the same groups. AFLP groups generated by the QDA model classified only the two isolates used to construct the model into VCG4 and VCG6, respectively; no isolates without a previous VCG designation were placed into either of these groups. Although AFLP cluster analysis did not classify any isolates into group 4, 25 isolates were classified into group 6. As previously mentioned, it is clear from the cluster analysis that group 6 displayed a very unique AFLP banding pattern, separating it from all other VCGs. Although the QDA model classified these 25 isolates as AFLP5, cluster analysis indicated these isolates were most closely related to group 6. It is very likely that the limiting factor was the small number of isolates of NA-VCG4 and NA-VCG6 available to build the model. In addition, cluster analysis was based on all of the polymorphisms, not just a few as in the case of QDA. Aside from group 6, the QDA model was useful in assigning a presumptive VCG to 99 isolates not analyzed for vegetative compatibility and also for those isolates that did not react with NA-VCG tester isolates (nonassigned). The fact that there was little change in frequencies of the NA-VCG groups, with the exception of NA-VCG5, after the addition of the AFLP samples provides evidence that the remainder of the predictions are accurate. The existence of isolates which do not anastomose with selected tester isolates could be explained by subgroups within larger VCGs, as has been demonstrated in *V. dahliae* (25,48).

Nitzan (36) investigated the pathogenicity of many of the isolates which were later evaluated by research reported here. As a group, isolates belonging to NA-VCG2 were more aggressive to potato than isolates assigned to NA-VCG3 based on sclerotia expansion on the stem, corresponding to the frequency at which isolates of these respective VCGs were recovered from potato samples. This could be an important differentiation of the agricultural significance for particular VCGs. Viji et al. (52) found some evidence of correlation between VCG and virulence in *Sclerotinia homoeocarpa*. Nitzan et al. (37) also found differences in aggressiveness among European *C. coccodes* isolates that correlated with VCG. Given that *C. coccodes* can infect multiple organs of the potato plant (17,31) and that VCGs can differ in aggressiveness, studies investigating the role this variability plays in disease development are warranted. For example, NA-VCG5 appears to be commonly associated with potato tubers. Because the tuber blemish phase is of worldwide importance (13,15,17,31), studies on global populations of *C. coccodes* recovered from tubers may prove enlightening. Preliminary results show that very little diversity is present in 27 *C. coccodes* isolates obtained from Scotland (14), England (9), and Ireland (4), with only VCG5 represented among these populations (N. C. Gudmestad, *unpublished data*). Additionally, one isolate originating from strawberry in Argentina was determined to be VCG6, corresponding to earlier results of isolates from non-potato hosts (N. C. Gudmestad, *unpublished*). Clearly, AFLP analysis will be a powerful tool in the study of global populations of *C. coccodes*.

The results reported here demonstrate the considerable genetic variation within *C. coccodes* populations and that the variation is structured into a limited number of subgroups. This, however, represents only North American isolates of *C. coccodes*. The worldwide distribution of this fungus mandates that the only way to obtain a true indication of its genetic diversity will be to analyze similar groups of isolates from throughout the world. Some work has been done on European and Israeli isolates that indicates a similar limited number of VCGs (37). North American and European isolates of *C. coccodes* have not yet been found to be complimentary in VCG analyses (36); therefore, these studies can be performed only using AFLP analysis. Additionally, VCG determination is limited by the ability to generate *nit* mutants to all isolates, and by an isolate's complementation ability. A significant percentage (22%) of the isolates studied here and elsewhere (36,37) do not anastomose, limiting the usefulness of VCG analysis. Conversely, AFLP analysis is not limited in its ability to determine relationships among *C. coccodes* populations. AFLP analysis, although expensive and technically complex, can evaluate a large population fairly efficiently and may provide a more rapid and definitive measure of the genetic relationships of worldwide populations. With *C. coccodes*, there are a number of bands that appear to be distinctive for a given VCG. Thus, these bands could

be used to develop VCG-specific sequence-characterized amplified region markers (10,55), as was done for a single RAPD-derived marker for a velvetleaf specific strain of *C. coccodes* (14). These are PCR-amplifiable and specific DNA segments which could be used for VCG-specific PCR testing. This would greatly simplify and expedite vegetative compatibility assignment and allow for more detailed investigations on the biology, ecology, and epidemiology of *C. coccodes*, thereby significantly adding to the body of information that currently exists (2,6,15,18,26,27).

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