Am. J. Potato Res. (2014) 91:75–87
DOI 10.1007/s12230-013-9331-2

Genetic Diversity of a Global Population of *Colletotrichum Coccodes* Using Amplified Fragment Length Polymorphism Markers

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Published online: 17 July 2013

Abstract The present study tested the hypothesis that the global *C. coccodes* population is genetically differentiated by region of origin. A total of 855 isolates, originating from North America (475), Europe (147), Israel (66), Australia (86), South Africa (14) and additional testers (67), which previously were assigned to VCGs (21 international VCGs) were examined for their genetic diversity. Using the previously assigned North American VCGs as the leading sub-population, and with the aid of amplified fragment length polymorphism (AFLP) markers, the isolates were grouped into five VCG/AFLP sub-populations (1, 2, 3, 4/5 and 6/7). These were distributed between two phylogeny clusters, designated Cc-A and Cc-B. Cluster Cc-A was unique and composed of isolates from VCG/AFLP6/7 only, whereas cluster Cc-B was composed of the remaining four VCG/AFLP sub-populations, which was further divided into 17 sub-clusters. VCG/AFLP4/5 was the most common VCG globally, followed by VCG/AFLP2. Most of the variation among the four geographic regions originated from the within population differentiation (84 %). It is concluded from these studies that the global population of *C. coccodes* exists as one large population with five main VCG/AFLPs worldwide and that they are probably of the same origin, however, geographic isolation caused these populations to differentiate and form distinct sub-clusters.

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Resumen En el presente estudio se probó la hipótesis de que la población global de *C. coccodes* esta diferenciada genéticamente por región de origen. Se examinaron para su diversidad genética un total de 855 aislamientos, originarios de Norteamérica (475), Europa (147), Israel (66), Australia (86), Sudáfrica (14) y otros adicionales (67), los cuales fueron asignados previamente como VCGs (21 VCGs internacionales). Utilizando la asignación previa de VCGs Norteamericanos, como la subpoblación de referencia, y con la ayuda de marcadores de polimorfismo de longitud de fragmentos amplificados (AFLP), se agruparon los aislamientos en cinco subpoblaciones VCG/AFLP (1, 2, 3, 4/5 y 6/7), que se distribuyeron entre dos grupos poligénicos, designados como Cc-A y Cc-B. El agrupamiento Cc-A fue único y se constituyó de aislamientos de VCG/AFLP6/7 únicamente, mientras que el grupo Cc-B se compuso de las restantes cuatro subpoblaciones VCG/AFLP, que a su vez fue dividido posteriormente en 17 sub-agrupamientos. El VCG/AFLP4/5 fue el más común globalmente, seguido por VCG/AFLP2. La mayor variación entre las cuatro regiones geográficas se originó de la diferenciación interna de la población (84 %). Se concluye de estos estudios que la población global de *C. coccodes* existe como una población grande con cinco principales VCGAFLPs en todo el mundo, y que son, probablemente, del mismo origen. No obstante, el aislamiento geográfico ha hecho que estas poblaciones se diferencien y formen diferentes sub-agrupamientos.

Keywords *Colletotrichum coccodes* · Vegetative compatibility groups · Population genetics · Population structure · Geographic origin

Introduction

Colletotrichum coccodes (Wallr.) Hughes is a cosmopolitan pathogen (Dillard 1992). It is primarily found on vegetables

in the *Solanaceae*, *Cucurbitaceae*, and *Fabaceae* families (Dillard 1992). On potato, *C. coccodes* causes black dot, a multifaceted disease with an array of symptoms affecting multiple organs.

C. coccodes is an imperfect fungus (Cano et al. 2004), thus vegetative compatibility may serve as a means of genetic exchange and be useful for measuring diversity (Cole and Kendrick 1981). Vegetative compatibility refers to the ability of an individual fungus to undergo mutual hyphal anastomosis and form viable heterokaryons. The potential for two fungal strains to undergo hyphal anastamosis is most commonly assessed through analysis of nitrate non-utilizing (*nit*) mutants. Strains that are vegetatively compatible with one another are described as members of the same vegetative compatibility group (VCG) (Leslie 1993). In the case of *C. coccodes* nit mutants are the only means for the characterization of VGCs via hyphal anastomosis.

Characterization of *C. coccodes* populations has been studied using *nit* mutants in many regions (Ben-Daniel et al. 2010; Nitzan et al. 2002, 2006; Shcolnick et al. 2007). Eight VCGs were identified from a Europe/Israel population (Nitzan et al. 2002; Shcolnick et al. 2007), seven VCGs were identified from a North American population (Nitzan et al. 2006), and six VCGs were identified from an Australian population (Ben-Daniel et al. 2010). Although the vegetative compatibility grouping method is widely used to study genetic diversity among populations of asexually reproducing fungi, it has certain limitations that can reduce its effectiveness and utility. Such limitations include the inability of some isolates to form *nit* mutants on chlorate medium (Strausbaugh et al. 1992) and that some mutant isolates are not able to anastomose with selected tester strains and form stable heterokaryons (Joaquim and Rowe 1990). The limitation of vegetative compatibility grouping to study variability among *C. coccodes* isolates from different continents is that there is very limited complementation among North American, Australian, and Europe/Israel isolates (Ben-Daniel et al. 2010; Nitzan et al. 2006). As a result, other methodologies are needed to study the diversity of a global *C. coccodes* population.

Variation in aggressiveness, morphological traits (Aqeel et al. 2008), and physiological traits (Nitzan et al. 2006) have been detected among the VCGs of *C. coccodes*. Isolates belonging to NA-VCG2 (Aqeel et al. 2008; Nitzan et al. 2006), NA-VCG5 (Nitzan et al. 2006) and NA-VCG6 (Aqeel et al. 2008) have been found to be the most aggressive in North America. In other regions of the world, *C. coccodes* isolates belonging to Eu/I-VCG5 for Europe/Israel (Shcolnick et al. 2007), and AUS-VCG4 for Australian population (Ben-Daniel et al. 2010) are the most aggressive. Among the studies investigating the aggressiveness of *C. coccodes* populations, aggressiveness has been assessed differently. In NA-populations; it was assessed based on tuber weight reduction, conidial and microsclerotial size and density production, total yield weight, and number of infected progeny tubers (Aqeel et al. 2008; Nitzan et al. 2006). In European/Israeli populations, aggressiveness was assessed based on the colonization of the potato different stem parts with *C. coccodes*. In contrast, for the Australian population aggressiveness of isolates was compared using necrotic lesion size on mature green tomato fruits (Ben-Daniel et al. 2010). It would be useful if aggressiveness assessment parameters for *C. coccodes* in potato were standardized worldwide to facilitate useful comparisons among isolates and cultivars. Regardless, it is not known at this time whether the most aggressive VCGs on each continent are genetically related. Such knowledge could facilitate the development of germplasm with resistance that would be stable regardless of where it is grown in the world.

Amplified fragment length polymorphism (AFLP) was efficient in studying relationships within and among North American VCGs of *C. coccodes* (Heilmann et al. 2006). With this method, five AFLP groups were separated within *C. coccodes*, coinciding almost exactly with the VCGs. Additionally; a relationship of specific AFLP bands to corresponding VCGs was reported using AFLP analysis. In the studies reported here, we hypothesize that the global *C. coccodes* is genetically differentiated by region. The main objectives of this study were (i) to determine if the most aggressive VCGs around the world are related by studying the global genetic diversity of *C. coccodes* populations including North American, European, Israeli, Australian, and South African isolates using AFLP markers, and to (ii) determine whether geographic origin is a factor in the genetic diversity of *C. coccodes*.

Materials and methods

C. coccodes isolates A total of 855 isolates, originating from North America (475), Europe (147), Israel (66), Australia (86), South Africa (14) and additional testers (67), which previously were assigned to VCGs (21 international VCGs) were examined for their genetic diversity. The North American population included 370 isolates collected from 2006 to 2009 (Gudmestad lab) that have not been previously studied and 105 previously assigned isolates (Heilmann et al. 2006). The unassigned North American isolates, were collected from different states (n=9), different fields within the state, and different potato plants within the field, and in some occasions different plant parts within the plant.

For recently collected isolates, pure cultures were obtained by transferring hyphal tips (five per isolate) to water agar. After 4–6 days, hyphal tips were excised from the water agar plates and transferred to 1 % PDA and streptomycin. The purified cultures were checked for contamination after 5 to 7 days. These steps were performed to remove the mycoparasite *Acremonium*

strictum and other contaminants which were occasionally present (Rivera-Varas et al. 2007). For long term storage, four ml of 7.5 % sterile skim milk solutions was added to 14 day old culture plates, sclerotia were gently scraped from the plate surface, and were used to establish stock solutions on silica gel crystals and stored at −80 °C (Smith 2005). *C. coccodes* isolates from Australia, Europe/Israel, and South Africa were received as cultures on PDA or as sclerotia grown in sterilized soil. These isolates were sub-cultured, hyphal tipped, and preserved on stock solutions on silica gel crystals and stored at −80 °C. Most of the isolates in this study originated from potatoes; 240 isolates from stems, 104 from tubers, 14 from roots, 9 from leaves, and the rest were as cultures with no known host tissue origin.

DNA extraction Pure cultures of *C. coccodes* were grown in Richard's solution (Xu et al. 2001), filtered through Whatman no. 1 filter paper (90 mm: Whatman Specialty Products, Inc) using a vacuum pump, frozen at −80 °C for 30 min, lyophilized for 24 h, and ground to a fine powder in liquid nitrogen using pre-cooled sterile mortar and pestle. DNA extraction was performed using a cetyltrimethyl ammonium bromide (CTAB) DNA extraction protocol (Doyle and Doyle 1987). DNA quantity and purity was assessed using a TD-700 Fluorometer (Turner Designs, Sunnyvale, CA) and agarose gel visualization (Alananbeh 2010). DNA quantities were diluted to be in a range of 50 to 70 ng μl^{-1}.

PCR assays PCR was performed on DNA from the newly collected isolates from North America, and for isolates obtained from Australia, Europe, Israel, and South Africa to further ensure species identity by using *C. coccodes* specific primers Cc1NF1 and Cc2nR1 (Cullen et al. 2002). *Colletotrichum lindemuthianum* and blank water were used as a negative control while one of the tester *C. coccodes* strains of North America was used as positive control. All *C. coccodes* isolates used in this study yielded the species-specific 349 bp polymorphic DNA band (Cullen et al. 2002) confirming their identity.

PCR amplification with universal primers ITS4 and ITS5 (White et al. 1990) was carried out on 26 *C. coccodes* isolates representing different geographic regions and VCGs. PCR amplicons were extracted, sequenced, and BLASTn via the NCBI database (www.ncbi.nlm.nih.gov). Sequences were submitted to the Gene Bank and accession numbers were obtained (Table 1).

AFLP assays AFLP reactions were performed according to the method of Vos et al. (Vos et al. 1995) as modified by Heilmann et al (2006) for *C. coccodes*. The AFLP product was visualized using a DNA analyzer, LI-COR model 4300. Default AFLP program was used to run the gels. It was programmed to run for 3.5 h, at 1,500 V (40 W).

Table 1 Accession numbers for 26 representative *C. coccodes* isolates from different geographic regions and vegetative compatibility groups using the Internal Transcribed Spacer Region ITS4 and ITS5 (White et al. 1990)

Accession #	Isolate ID	Origin	Host	VCG
JX205169	NE04-83-4	USA	Potato	VCG/AFLP1
JX205170	NV04-66-5	USA	Potato	VCG/AFLP1
JX205171	C237iL	USA	Potato	VCG/AFLP1
JX205172	C116iL	USA	Potato	VCG/AFLP2
JX205173	OR04-61-7	USA	Potato	VCG/AFLP2
JX205174	C244iL	USA	Potato	VCG/AFLP2
JX205175	WA-13	USA	Potato	VCG/AFLP3
JX205176	ORG-9C	USA	Potato	VCG/AFLP3
JX205177	OR04-53-2	USA	Potato	VCG/AFLP3
JX205178	C43iL	USA	Potato	VCG/AFLP4/5
JX205179	MI04-70	USA	Potato	VCG/AFLP4/5
JX205180	OR04-62-3	USA	Potato	VCG/AFLP4/5
JX205181	C210	USA	Potato	VCG/AFLP6/7
JX205182	NE04-67	USA	Potato	VCG/AFLP6/7
JX205183	CcMI04-70	USA	Potato	VCG/AFLP6/7
JX205184	SCRI-C33	USA	Potato	VCG/AFLP6/7
JX205185	C54	USA	Potato	VCG/AFLP6/7
JX205186	Cc08NE-3	USA	Potato	VCG/AFLP6/7
JX205187	AUS-6	Australia	Potato	VCG/AFLP4/5
JX205188	AUS-98/07AH	Australia	Potato	VCG/AFLP4/5
JX205189	SA-H1	South Africa	Potato	VCG/AFLP1
JX205190	SA-K14	South Africa	Potato	VCG/AFLP1
JX205191	SA-R6	South Africa	Potato	VCG/AFLP6/7
JX205192	Si-86	Israel	Potato	VCG/AFLP4/5
JX205193	Si-130	Israel	Potato	VCG/AFLP4/5
JX205194	Si-168	Israel	Potato	VCG/AFLP4/5

Scoring of AFLP fragments AFLP images were scored for presence or absence (1=presence or 0=absence) of a single band. The bands for each isolate were scored manually. All DNA fragments within the range of 50 to 650 bp were scored for the three primer sets, *Eco*-AC/*Mse*-CC, *Eco*-AG/*Mse*-CC, and *Eco*-AT/*Mse*-CC for each isolate, then combined and used to develop a binary matrix. A total of 210 AFLP fragments were obtained from the 855 isolates of *C. coccodes*, including 71 DNA fragments with *Eco*RI-AC/*Mse*I-CC, 66 with *Eco*RI-AG/*Mse*I-CC, and 73 with *Eco*RI-AT/*Mse*I-CC primers. The matrices with scores for the presence or absence of bands contained 180180, 56628, and 62634 data points for the three primer sets, respectively. The three primer sets used in this study generated 210 putative loci. The AFLP profiles were highly polymorphic among *C. coccodes* isolates. Polymorphism of the three primers was high, 98.6 %, 100 %, and 93.2 % for *Eco*RI-AC/*Mse*I-CC, *Eco*RI-AG/*Mse*I-CC, *Eco*RI-AT/*Mse*I-CC respectively. The population differentiation estimate G_{ST} was

consistent among the three primer pairs and was 0.29, 0.27, and 0.30 for *Eco*RI-AC/*Mse*I-CC, *Eco*RI-AG/*Mse*I-CC, *Eco*RI-AT/*Mse*I-CC, respectively. Therefore, data from all three primer pairs were combined for further analysis.

AFLP reactions were performed on DNA of *C. coccodes* tester isolates (n=40) and representatives of isolates from the five regions (n=48) five times during the study to ensure that banding patterns were consistent from run to run and to detect the presence of homoplasy. DNA extraction and AFLP reactions were done separately each time. AFLP banding pattern was consistent in each run for all *C. coccodes* isolates tested when repeated.

Population cluster analysis The *C. coccodes* population structure was analyzed by two methods (i) Phylogeny Inference Package (PHYLIP version 3.69, University of Washington) along with Statistical Analysis System (version 9.2; SAS Institute; Cary, NC), and (ii) principal coordinate analysis (PCoA) via GenAlex 6.3 software (Peakall and Smouse 2006).

(i) *Cluster analysis using PHYLIP and SAS*. Cluster analysis was generated based on an isolates relationship to known NA-VCGs since the AFLP method of analysis was developed using isolates from this region (Heilmann et al. 2006). In PHYLIP, SEQBOOT, NEIGHBOR, CONSENSE, and DRAWGRAM programs were used however, these programs do not allow bootstrap values to be generated on the final dendogram. For bootstrapping, SAS was used to generate the bootstrap values on the tree nodes. Preliminary analysis showed that the dendogram generated by PHYLIP had few clusters with non-assigned isolates. These non-assigned isolates formed individual clusters containing one or more isolates in each. Further analysis using POPGENE V1.32 (Yeh et al. 1997) was conducted to study the genetic similarity among the clusters in order to assign the isolates into their designated VCGs. Additionally, analysis of molecular variance among and within clusters was conducted using GenAlex 6.3 software (Peakall and Smouse 2006).

(ii) *Principal Coordinate Analysis* (*PCoA*). Principal coordinate analysis (PCoA) based on Euclidean distance matrix of the global *C. coccodes* population (n=855) was performed using GenAlex 6.3 (Peakall and Smouse 2006). Euclidean distance was computed using Change MVSA into Multivariate Surface Analysis (MVSA) (Kovach 2007).

Tester strains were used in both cluster analysis to study how isolates of *C. coccodes* assigned into their presumptive VCGs.

Population genetic analysis The *C. coccodes* isolates excluding the tester strains (n=788) were classified into five regions based on geographic origin: North America (NA), Australia (AUS), Europe (EU), Israel (I), and South Africa (SAf). Although the Europe/Israel (EU/I) population of *C. coccodes* was originally described as a single population (Nitzan et al. 2002), for the purpose of this analysis we handled isolates from each region separately to test that assumption. Further analysis was performed for geographic differentiation. Data generated from the 210 loci for *C. coccodes* isolates were combined and analyzed using multiple softwares: POPGENE version 1.32 (Yeh et al. 1997), GenAlex 6.3 software (Peakall and Smouse 2006), MVSA software (Kovach 2007), and Multilocus 1.3 b software (Agapow and Burt 2001). Genetic testing included multilocus genotype (G); Nei's gene diversity (h); number and percentage of polymorphic loci; H, which is a function of the number and frequency of alleles at each locus was estimated in the within population (H_S) and the total population (H_T); G_{ST}, the population differentiation across the 210 loci; analysis of molecular variance (AMOVA); number of different genotypes (G), genotypic diversity (GD), linkage disequilibrium (LD); number and percentage of polymorphic loci; and pairwise population matrix of Nei genetic distance and identity. Genotypic diversity versus number of loci was also tested using Multilocus 1.3b software for each population and for the 788 isolate *C. coccodes* population to see whether scoring more loci would increase the genotypic diversity, or whether it has reached a plateau.

For AMOVA, the population based on geographic origin was divided into four regions; North American, European/Israeli, Australian, and South African. The number of regions was chosen based on a previously generated dendogram using Nei's genetic distance and the UPGMA method with 1,000 bootstraps generated by POPGENE (version 1.32) (Yeh et al. 1997). AMOVA analysis was based on the pairwise squared Euclidian distances generated via MVSA software (Kovach 2007), and was conducted via GenAlex 6.3 (Peakall and Smouse 2006). The variance was partitioned into three covariance components Phi (Φ) fixation indices (ΦPR, ΦRT, and ΦPT). The level of significance for those indices was (P<0.05), and was determined using 1,000 permutations (Excoffier et al. 1992).

In some of the analyses conducted (h, H_T, H_S, G_{ST},), polymorphic loci number and percentage, the population structure among the five regions was studied in two ways. First, data from all of the 788 isolates (without tester isolates) were considered as one population to quantify H_T, H_S, and G_{ST}, AMOVA and other statistics. Second, data for each population pair (NA vs AUS, NA vs Eu, NA vs I, NA vs SAf, AUS vs Eu, AUS vs I, AUS vs SAf, Eu vs I, Eu vs SAf) were statistically tested using POPGENE V 1.32 (Yeh et al. 1997).

Pairwise comparisons among the five geographic origins means of *C. coccodes* were tested by Tukey-Kramer multiple comparisons test using PROC ANOVA in SAS software for the binary matrix.

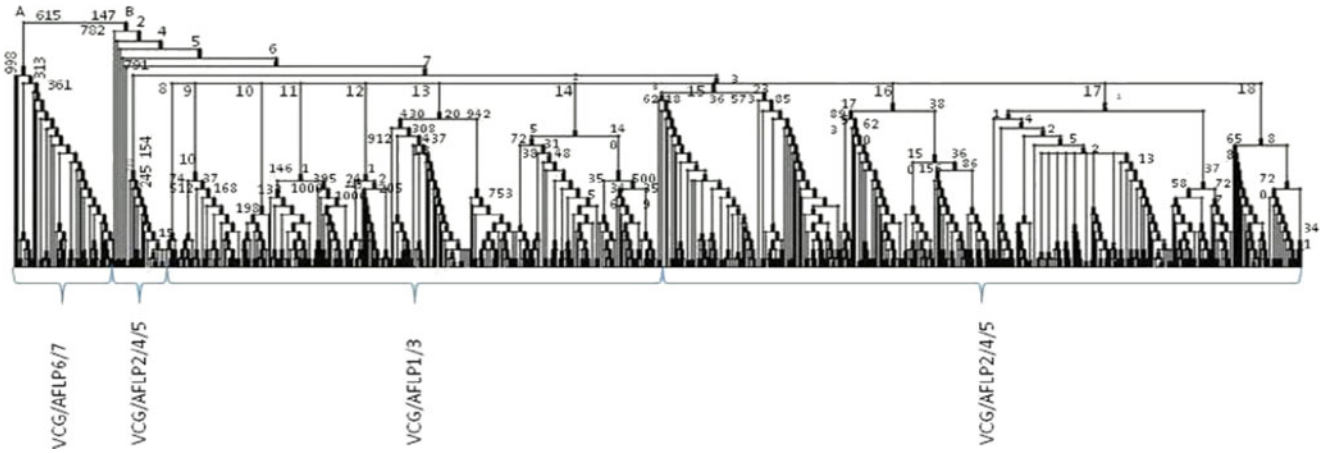

Fig. 1 Cluster analysis of the global *C. coccodes* populations. SAS along with PHYLIP package were used to generate the 1,000 bootstraps and the consensus tree respectively. The NEIGHBOR program in PHYLIP was used to generate a tree for each bootstrap sample using the Neighbor-Joining method of tree construction with UPGMA clustering. Three primer pairs were used to generate the binary matrix for further data analysis: *Eco*RI-AG/*Mse*I-CC, *Eco*RI-AC/*Mse*I-CC, and *Eco*RI-AT/*Mse*I-CC. Cluster analysis was generated based on an isolates relationship to known NA-VCGs

Results

Statistical analysis and clustering

Cluster analysis using the PHYLIP and SAS Cluster analysis based on 1,000 bootstraps and UPGMA separated *C. coccodes* isolates into two main clusters, one had only isolates belonging to VCG/AFLP6/7 (cluster A with 615 bootstrap value) and the other (cluster B with 147 bootstrap value) included all other isolates in the different VCG/AFLPs (Fig. 1 and Table 2). The first cluster included VCG/AFLP6/7 tester strains (n=13) and 52 other isolates (Table 2). North American isolates belonging to VCG/AFLP6 and VCG/AFLP7 could not be separated and all isolates in these two AFLP/VCGs clustered together and were distinct from other isolates. VCG/AFLP7 isolates formed a distinct sub-cluster within the VCG/AFLP6/7 cluster. The isolates in this group included 28 previously assigned North American isolates, 14 recently collected isolates from North America, as well as nine isolates from South Africa, and one isolate from Scotland (Table 2). The nine South African isolates that clustered with VCG/AFLP6/7 were separated also from the other isolates into a distinct sub-cluster. These nine isolates amplified a sequence characterized amplified region (SCAR) primer, AGb6F/R, designed to be specific for VCG/AFLP6/7 (Alananbeh 2010). All *C. coccodes* isolates originating from hosts other than potato clustered with this VCG/AFLP.

A total of 17 sub-clusters were formed within the second main cluster (cluster B) (Fig. 1). However, sub-clusters B1–B5 had 1, 3, 1, 3, 1 isolates, respectively, and were combined into two sub-clusters for future analysis using additional genetic analysis software.

The VCG/AFLP1 was within cluster B and formed sub-clusters B1–B9 (Fig. 1). This cluster included 104 isolates, 95 of them were from USA, five from South Africa, three isolates from Europe, and one from Australia. Out of the 95 isolates from North America, 19 isolates were previously assigned as VCG/AFLP1, 70 new previously unassigned *C. coccodes* isolates from the USA, and the six North American tester isolates (Table 2).

VCG/AFLP3 was distinct from the other groups and formed one sub-cluster (B10) (Fig. 1) that included 14 previously assigned VCG/AFLP3 isolates, 15 AUS-VCG testers, 11 EU/I VCG testers, and 11 North American tester isolates (Table 2). No isolates recently collected from the United States clustered within this group.

C. coccodes isolates belonging to VCG/AFLP4/5 and VCG/AFLP2 formed the majority of the sub-clusters in cluster B; sub-clusters B11–B14 and B15–B17, respectively (Fig. 1). For VCG/AFLP2, 286 isolates were assigned. There were 256 NA-isolates, 24 previously assigned isolates, and six NA-tester isolates (Table 2). VCG/AFLP5 sub-cluster was the largest cluster and included 338 isolates belonging to Europe/Israel (n=203), Australia (n=85), North America (n=30), previously assigned (n=20), and 10 NA-tester isolates. Both, the Australian and the Europe/Israel isolates formed distinct sub-clusters. Australian isolates were in sub-cluster B13, and the Europe/Israel isolates were in sub-clusters B12 and B14.

Excluding the tester isolates, VCG/AFLP4/5 was the most common VCG globally, containing 338 of the 788 isolates assigned, followed by VCG/AFLP2 containing 286 isolates

Table 2 Summary of the cluster analysis of the global *C. coccodes* populations using PHYLIP and SAS with AFLP three primer pairs and 1,000 bootstraps

Origin[a]	Organ[b]	VCG/AFLP[c]	# of isolates	Cluster #[d]
USA[e]				
n=475	L,R,S,St,T	1	89	B (1–4,6,8,9)
		2	280	B (15–17)
		3	14	B (10)
		4/5	50	B (11,13,17)
		6/7	42	A
Europe[f]	Sc	1	3	B (8)
n=147		2	6	B (16)
		3	0	
		4/5	137	B (12,14)
		6/7	1	A
Israel	Sc	1	1	B5
n=66		2	0	–
		3	0	–
		4/5	65	B (12,14)
		6/7	0	–
Australia[g]	C	1	1	B (6)
n=86		2	0	–
		3	0	–
		4/5	85	B (13)
		6/7	0	–
South Africa	C	1	5	B (7)
n=14		2	0	–
		3	0	–
		4/5	0	–
		6/7	9	A
Total			855	

[a] USA isolates obtained from Dr, N, C, Gudmestad laboratory, ND, USA, European, Israeli, Australian, and South African isolates were received as cultures from Dr, Leah Tsror, Israel. All the isolates received from Europe/Israel had no information about the VCG to which they belong using *nit* mutant technique

[b] The organ from which the *C*, *coccodes* was isolated, L: leaf, R: root, S: stem, St: Stolon, T: tuber, Sc: isolates were received as sclerotia preserved on sterilized cork, and C: isolates were received as cultures grown on potato dextrose agar

[c] 67 tester strains were used in this study including 40, 16, and 11 strains for the North American, Australian, and Europe/Israel *C. coccodes* population, respectively. The tester strains used in this study were composed of multimember isolates representing the different VCGs for each continental population. Isolates of *C. coccodes* from South Africa have been found to be vegetatively incompatible with all known tester strains from the other geographic regions (L. Tsror, unpublished). North American testers were obtained as sclerotia on sterilized soil from Johnson and Miliczky 1993

[d] Based on cluster analysis, five main VCG/AFLP's were separated; VCG/AFLP1, VCG/AFLP2; and VCG/AFLP3, VCG/AFLP4/5, and VCG/AFLP6/7. Isolates were assigned based on their clustering with known NA-VCG-tester *C*, *coccodes* strains. There were two main clusters (A and B) (Fig. 1), and 17 sub-clusters within cluster B. Cluster analysis was conducted via PHYLIP and the SAS software to analyze AFLP data of 855 *C*, *coccodes* isolates, Unweighted pair group method with arithmetic mean and 1,000 bootstraps were used to generate a dendogram using a similarity matrix of 210 AFLP bands that were scored as present or absent

[e] Isolates from USA were originated from nine states, CO (*n*=22), MI (*n*=13), Minnesota (*n*=80), Montana (*n*=11), North Dakota (*n*=86), Nebraska (*n*=28), Nevada (*n*=19), Texas (*n*=24), Wisconsin (*n*=87), and previously assigned isolates (*n*=105), these isolates were characterized to VCG's using AFLP by Heilmann *et al* (2006). Most of the previously assigned *C. coccodes* isolates were isolated from potato; only two isolates were from red pepper, five from tomato, and three from peppermint

[f] Isolates from Europe were originated from six countries; England (*n*=9), France (*n*=9), Germany (*n*=3), Ireland (*n*=4), Netherland (*n*=57), and Scotland (*n*=65). All the isolates from England, France, Germany, and Ireland clustered with VCG/AFLP4/5

[g] Australian isolates were collected from 12 different locations in Australia (Ben-Daniel et al. 2010)

(Table 2). On the other hand, VCG/AFLP3 was the least common group globally (Table 2).

Cluster analysis assigned *C. coccodes* into their presumptive VCGs. However, genetic similarities among the clusters were used to assign the sub-clusters with the ones that had the NA-testers. This was performed by analyzing the data using the POPGENE software, where the genetic similarities among the 15 clusters were calculated. A cluster with a genetic similarity of more than 80 % was combined with the others including the VCG tester strains. Differentiation analysis among the 15 clusters was (50 %). As a result of these findings, further testing by principal coordinate analysis (PCoA) was conducted.

Principal Coordinate Analysis (PCoA) The scree plot generated by PCoA demonstrated that data variability is represented by the first five axes of the ordination based on Kaiser's rule which states that the minimum eigenvalue should be the average of all eigenvalues (Kaiser 1960). Therefore, the first three axes explained 42.01 %, 19.98 %, and 14.85 % of the variability of the *C. coccodes* population tested, respectively. When examining the first three axes (Fig. 2), it is clear from the scatter plot of axis one versus axis two and axis one versus axis three that *C. coccodes* isolates belonging to VCG/AFLP6/7 were very distinct from the rest of the population regardless of their geographic origin and that other VCG/AFLPs were together in a single but separate group. These results are consistent with the PHYLIP method of cluster analysis. Isolates that originated from different geographic origins could be found intermingled with NA isolates (Fig. 2). Based on this analysis, VCGs are distinct but not among geographic origins (Fig. 2) as *C. coccodes* isolates from the five geographic origins are mixed. However, Tukey-Kramer multiple comparison tests on mean values among the five geographic origins demonstrates that the North American population is the only one that is significantly different from the others (Table 3).

Genetic structure of global C. coccodes population based on geographic origin Genetic diversity of the 788 *C. coccodes* isolates, excluding the tester isolates, among the five geographic regions was analyzed using the 210 loci. Total genetic diversity was relatively moderate (H_t=0.22) and gene diversity within a regional population was relatively low (H_S=0.15) (Table 4). The overall differentiation among populations was relatively high (G_{ST}=0.30). This value was also

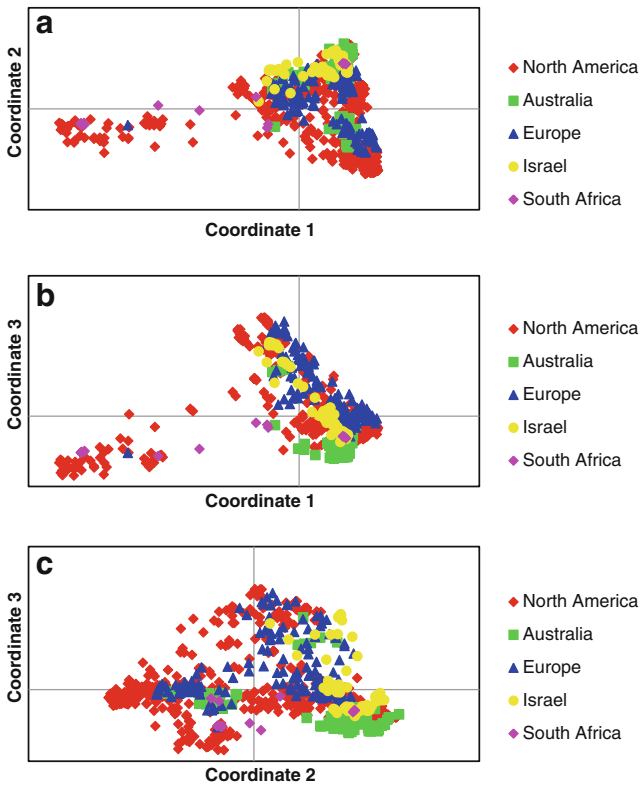

Fig. 2 Two dimensional principal coordinate plots of a matrix of Euclidean distances for 855 *C. coccodes* collected mainly from potato from five geographic origins. The first, second, and third principal coordinates account for 42.01 %, 19.98, and 14.85 % of the variation, respectively

Table 3 Tukey-Kramer multiple comparison results among the five geographic origins of the global *C. coccodes* isolates

Source	df	*F*-value	Pr>*F*
Model	4	148.09	<.0001
Error	849		
Corrected	853		
Pop comparison[a]	Between means	*P*[b]	
NA–Eu	178.88	*	
NA–AUS	206.49	*	
NA–I	224.49	*	
NA–SAf	250.49	*	
Eu–AUS	27.62	NS	
Eu–I	45.62	NS	
Eu–SAf	71.62	NS	
AUS–Eu	−27.62	NS	
AUS–I	18.00	NS	
AUS–SAf	44.00	NS	
I–Eu	−45.62	NS	
I–AUS	−18.00	NS	
I–SAf	26.00	NS	
SAf–Eu	−71.62	NS	
SAf–AUS	−44.00	NS	
SAf–I	−26.00	NS	

[a] Populations compared based on their geographic origin; NA: North America, AUS: Australia, Eu: Europe, I: Israel, and SAf: South Africa

[b] Comparisons significant at the 0.05 level are indicated by * and the non-significant as NS

Table 4 Genetic variation statistics for the 210 loci of *C. coccodes* based on geographic differentiation

Population	Sample size	#Pol. Loci[a]	%[b]	h[c]	*G*[d]	*GD*[e]	LD[f]
All pop.	788[g]	198	94.29	0.22	–	–	–
North America	475	193	91.90	0.23	475	0.99	0.08
Australia	86	92	43.81	0.11	82	0.99	0.09
Europe	147	149	70.95	0.10	113	0.99	0.07
Israel	66	96	45.71	0.09	53	0.98	0.07
South Africa	14	113	53.81	0.21	14	1.00	0.22

[a] The number of polymorphic loci

[b] The percentage of polymorphic loci

[c] h=Nei's (1973) gene diversity

[d] *G*: number of distinct genotypes

[e] *GD*: genotypic diversity

[f] Measurement of linkage disequilibrium (LD), all values are significant from zero ($P<0.01$)

[g] Only the *C. coccodes* isolates were used in the analysis, tester strains were not included

similar to the mean G_{ST} value obtained from merging the three primer pairs.

Overall gene diversity of the total population studied was relatively moderate (0.22). It ranged between 0.09 (low) in Israel to 0.23 (moderate) in North America (Table 4). The number of distinct genotypes and genotypic diversity of *C. coccodes* was high in all regions (Table 4). There were 737 distinct genotypes recognized out of 788 isolates. Genotypic diversity ranged from 0.98 to 1.00. When plotting the genotypic diversity versus the 210 loci used in this study, genotypic diversity reached a plateau, indicating that these markers were adequate in estimating the existing diversity (Fig. 3). LD was low among the five regions, ranging from 0.07 to 0.09, except in South Africa where LD was 0.22. Although linkage disequilibrium values were low, they were all significantly different from zero ($P<0.01$) (Table 4).

Pairwise comparisons of population genetic identity and genetic distance among the five regions demonstrated that the European and Israeli population pair had the highest genetic similarity (0.99) (Table 5), while South Africa had the least similarity with any of the other regions (Table 5). The overall estimated differentiation value among the five regions was 0.30. It was expected to have high number of migrants between Israel and Europe due to the high genetic similarity among these two geographic regions (Table 5).

When analyzing the global population of *C. coccodes* by continent, the UPGMA Nei-based dendogram clustered the five regional populations into two main clusters (Fig. 1). The first cluster included *C. coccodes* isolates from North America, Australia, Europe, and Israel, and the second cluster had only *C. coccodes* populations from South Africa. Populations from Europe and Israel were more similar compared to the other populations (Table 6), confirming earlier descriptions of that population (Nitzan et al. 2002). North American and Australian populations were also distinct from the populations of Israel and Europe. Based on the UPGMA dendogram, populations were divided into four regions for AMOVA analysis, where both Israeli and European populations were considered as one region. AMOVA showed that there was 14 % variation originating from variation among regions (ΦRT, $P=0.001$), and only 2 % variation originated from among populations within regions (ΦPR, $P=0.001$). The variation originated from within the population estimated variance accounted for 84 % (ΦPT, $P=0.78$) (Table 7).

Discussion

The study reported here is the first attempt to investigate the global population of *C. coccodes* at the molecular genetics level using a molecular marker. Only one previous study has evaluated *C. coccodes* isolates using AFLP markers and that study only included isolates from North America (Heilmann et al. 2006). The study reported here uses a much larger population and includes isolates from five continents including North America, Australia, Europe, Israel (Asia), and Africa. The high number and percentage of the polymorphic loci obtained using AFLP markers demonstrated the

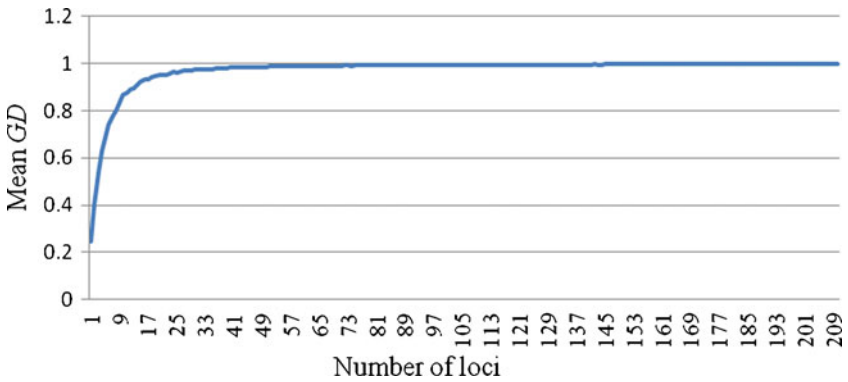

Fig. 3 Genotypic diversity versus number of loci. Analysis was conducted using Multilocus 1.3b software (Agapow and Burt 2001)

Table 5 Pairwise comparison matrix of Nei genetic identity (above) and Nei genetic distance (below) for *C. coccodes* populations from NA, AUS, Eu, Israel, and SAf based on their geographic origin

Pop ID[a]	NA	AUS	Eu	Israel	SAf
NA	****	0.94	0.95	0.93	0.85
AUS	0.06	****	0.95	0.94	0.83
Eu	0.05	0.05	****	0.99	0.82
Israel	0.07	0.06	0.01	****	0.82
SAf	0.16	0.19	0.20	0.20	****

[a] Population ID: NA: North America, AUS: Australia, Eu: Europe, Israe, Middle East, and SAf: South Africa. Each population was compared with the other populations (NA vs AUS, NA vs Eu, NA vs Israel, NA vs SAf, AUS vs Eu, AUS vs Israel, AUS vs SAf, Eu vs Israel, Eu vs SAf) using POPGENE version 1.32

effectiveness of these loci to differentiate the *C. coccodes* population efficiently and proved to be powerful in detecting genetic diversity in the global population of this plant pathogen. The data presented here clearly demonstrates that AFLP analysis is informative at the species level for *C. coccodes* as it has been for other fungal species (Kantardjiev et al. 2004; Mueller and Wolfenbarger 1999; Nusaibah et al. 2010; Savelkoul et al. 1999). Further data analysis using different genetic and statistical software was capable of studying and differentiating the global *C. coccodes* population effectively.

From the studies reported here, it appears that the global *C. coccodes* population is intermingled, however, they formed distinct sub-clusters or groups within the main clusters. The North American population had values of gene diversity (h) and polymorphic loci numbers and polymorphic percentages similar or greater than that found in the remainder of the global population, suggesting that the North American population is a mix of populations that are found

Table 6 Pairwise population matrix of average differentiation among populations (G_{ST})[a]) for *C. coccodes* population from NA, AUS, Eu, Israel, and SAf[b]

Pop ID	NA	AUS	Eu	Israel	SAf
NA	****				
AUS	0.13	****			
Eu	0.12	0.16	****		
Israel	0.15	0.19	0.03[c]	****	
SAf	0.21	0.30	0.32	0.33	****

[a] Estimate Nei's (1973) G_{ST}, average differentiation among populations

[b] Populations ID: North America (NA), Australia (Aus), Europe (Eu), Israel, and South Africa (SAf). Each population was compared with the other populations (NA vs AUS, NA vs Eu, NA vs Israel, NA vs SAf, AUS vs Eu, AUS vs Israel, AUS vs SAf, Eu vs Israel, Eu vs SAf)

[c] Gene flow between Israel and Europe are expected to be high due to the high genetic similarity and low differentiation between these two populations

Table 7 Analysis of molecular variance (AMOVA) for *C. coccodes* populations from five regions

Source of variation	df	Est. Var.	%	Φ Value[g]	*P*(rand>= data)
Based on one population[a]					
Among populations	4	0.52	15	0.15	0.001
Within populations (ΦPT)[b]	783	2.94	85		
Based on geographic origin[c]					
Among regions (ΦRT)[d]	3	0.49	14	0.14	0.001
Among populations/ regions (ΦPR)[e]	1	0.06	2	0.02	0.001
Within populations (ΦPT)[f]	783	2.94	84	0.16	0.001

[a] *C. coccodes* was considered as one population, so the variance was partitioned into among and within population for the first case

[b] PhiPT was calculated as the proportion of estimated variance for among populations, relative to the total estimated variance

[c] *C. coccodes* was partitioned into five populations; North America, Australia, Europe, Israel, and South Africa based on its geographic origin and four regions based on the genetic similarity between European and Israeli populations

[d] PhiRT was calculated as the proportion of the estimated variance of among regions, relative to the total variance

[e] PhiPR was calculated as the proportion of variance among populations within groups, relative to the variance among and within populations

[f] PhiPT was calculated as the proportion of estimated variance for among populations and among regions, relative to the total estimated variance

[g] Probability of obtaining low Phi values was determined by 1,000 permutations

elsewhere in the world and is likely due to a potato crop that moved worldwide from its center of origin. The South African population also had high values of gene diversity and is most likely due to the fact that most of the South African isolates belong to VCG/AFLP6/7, a group found to have the highest genetic diversity and highest genetic differentiation of any *C. coccodes* VCG. It is interesting to note that in vegetative compatibility studies, *C. coccodes* isolates from South Africa only generate *nit* mutants at a very low frequency (18 %), seldom forming complementation with other isolates while also being self-incompatible (L. Tsror, unpublished data). The South African isolates could only be grouped by using AFLP which then allowed us examine their relatedness to other VCG/AFLP groups. Perhaps more interesting is that there were VCGs found in only two geographic origins but no others. For example, VCG/AFLP2 was detected mostly in populations from North America with few isolates (n=6) from Europe, while VCG/AFLP6/7 was identified only in populations originating from North America and South Africa, clearly demonstrating a geographic pattern within the global *C. coccodes* population. Although difficult to explain, these differences may be due to environmental conditions, geography, and strong selection forces such as the pathogen-induced selection and differences in alternative

host plant diversity that may have a role in generating variability within populations, especially for pathogens having vegetative compatibility systems (Burdon and Silk 1997). In these species, conditions that support new genotype generation would also support the establishment of new VCGs, whether through gene flow or other forces (Burdon and Silk 1997). Furthermore, these factors could explain the limited complementation among the continental populations of *C. coccodes* found in previous studies.

Further confirmation of low genetic variation among populations was revealed in the AMOVA test and the common AFLP alleles that were shared across the five continents. Based on the geographic origin, most of the estimated variation was a result of within population variation (84 %) as there were high numbers of distinct genotypes, high genotypic diversity, and the existence of many VCGs within the one population. In a study conducted to find the genetic relationships for *Gibberella zeae* (Schwein.) among five hosts, 90 % of the variation in the fungus resulted from the within population (hosts) differentiation; and low LD values (0.003 to 0.04), significant from zero, were reported, suggesting that genetic drift or sample size are possible mechanisms for deviation from equilibrium (Burlakoti et al. 2008). In our study, LD values were low but significant from zero except for the South African population, where the sample size was small. Genetic drift could have effect on the *C. coccodes* population; the strong isolation between subpopulations could result from foundation effects. Furthermore, the low significant LD levels in our study suggests no random association of alleles and a possible cryptic sexual cycle (Comont et al. 2010) or evidence for clonal propagation (Farfán et al. 2002; Hsiang and Mahuku 2002). However, these hypotheses should be further studied using other markers and methods such as simple sequence repeat markers (SSR) and gene sequencing.

The *C. coccodes* population analysis showed strong evidence for a relatively moderate amount of genetic diversity for an asexual fungus and relatively high genetic differentiation among populations, indicating limited genetic exchange among the five regions. Expected distance between populations from different geographic origins would be large if there is a complete geographic specialization. However, the genetic distances from the five regions, North America, Australia, Europe, Israel, and South Africa, revealed that *C. coccodes* isolates from the five regions share a close relationship. Similarly, the lack of genetic variation among the five populations of *C. coccodes* confirms the presence of a large but genetically differentiated population due to the geographic isolation except between Israel and Europe where the differentiation was low due to genotype flow or migration of individuals from one geographic region to another (Horvarth and Vargas 2004). Seed potato tubers produced in Europe are sold and moved to Israel (Tsror Lahkim et al. 1999), explaining the low population differentiation and expected high genotype flow between these two regions.

We are cautious to make genetic inferences on *C. coccodes* isolates obtained from different hosts because of the imbalanced population samples where the majority of the isolates were recovered from potato but only few were from tomato, peppermint, and pepper. With this in mind, there are some interesting observations that can be made regarding non-potato *C. coccodes* isolates. Based on host differentiation, a high differentiation value was obtained among hosts. Isolates originated from tomato, red pepper, and peppermint were >94 % genetically identical to each other, while they were 72 % identical to the isolates from potato. This is not surprising since all the isolates (n=10) originated from tomato, red pepper, and peppermint belonged to VCG/AFLP6/7. A previous study showed that *C. coccodes* isolates originated from tomato were pathogenic on both tomato and pepper but with higher aggressiveness on the host from which they were recovered, suggesting a certain level of host specificity (Hadden and Black 1989). In another study, a *C. coccodes* isolate from eastern black nightshade was only pathogenic to its original host but not to potato, tomato, pepper and other hosts (Andersen and Walker 1985). Although there are few cross-inoculation studies using *C. coccodes* recovered from different hosts, these data are available for other plant pathogenic fungal species. Tomato strains of *V. dahliae* were found to be distinct from other strains recovered from other hosts with little admixture. In that study (Atallah et al. 2010), 22 SSR markers and 242 isolates were used from different hosts and genotype flow was observed among the different hosts except tomato. In the study reported here, more isolates representing different hosts, and using a co-dominant marker like SSR will assist in understanding the existence of differentiation among *C.coccodes* recovered from various hosts.

Although the populations of *C. coccodes* from different geographic origins were shown in the studies reported here to be very similar, these isolates are frequently not able to anastomose with each other (Ben-Daniel et al. 2010; Nitzan et al. 2006). Allelic compatibility, which is simply a system in which individuals have to be similar at all the vegetative compatibility (*vic*) or heterokaryon (*het*) loci in order to form stable heterokaryon, could explain the inability of these populations to anastomose with each other using the *nit* mutants technique. Our data suggest that populations of *C. coccodes* are closely related, but the geographic isolation provided a differentiating tool among the populations. All the isolates from the whole population were intermingled with the NA-population; however, sub-clustering of each geographic region in separate from the other regions was an indication of that geographic isolation. Furthermore, PCoA showed that global *C. coccodes* populations belonging to VCG/AFLP1, 2, 3, and 4/5 groups centered on the X and Y axis in the scatter plots indicating similarities found

between these groups. However, isolates belonging to VCG/AFLP6/7 were very distinct compared to the other VCGs. Therefore, we reject our original hypothesis that the global *C. coccodes* population is not closely related at the molecular genetics level. Furthermore, based on the data presented here, we further postulate that at one time, the *C. coccodes* population was isolated at the center of origin of potato and were all the same. In this hypothesis it is likely that *C. coccodes* became established on each continent as the potato was moved around the globe. Due to the lack of intermixing of the populations after their introduction, the *C. coccodes* population on a continent became bottle-necked which may explain the loss of vegetative compatibility that exists among some regional populations. This also would explain the existence of certain VCGs on some continents and not on others as revealed by our data.

The use of AFLP markers and a wide collection of isolates used in this study differentiated the global population and assigned the isolates into different groups regardless of origin, the VCG to which it belongs, and whether it can form a stable heterokaryon. Screening more primers to test this global population could provide additional differentiation among VCGs and populations (Cilliers et al. 2000). AFLPs are dominant and multilocus markers that proved to be useful in assessing genetic differences among populations, individuals, and species (Mueller and Wolfenbarger 1999). Based on our study, the population structure of *C. coccodes* isolates appear to have originated as one large population with five main groups (VCG/AFLP1, VCG/AFLP2; VCG/AFLP3; VCG/AFLP4/5; and VCG/AFLP6/7). However, the dominant character of AFLP marker limits their usefulness in viewing heterozygosity. Thus, using co-dominant markers which can detect heterozygosity alleles, such as SSR markers could provide additional information at the genetic level and could further differentiate the global *C. coccodes* population. SSR markers have been successfully used to study the phenotypic uniformity and outcrossing, and evolutionary and migratory histories of *Sclerotinia sclerotiorum* and *Verticillium dahliae* (Kleb.), respectively (Atallah et al. 2004; Atallah et al. 2010) and have also been used to examine the genetic variability and differentiation according to geography, host, and growing system in *Botrytis cinerea* Pers. from Tunisia (Karchani-Balma et al. 2008).

Variation in aggressiveness among *C. coccodes* VCGs within different regions has been reported. NA-VCG2, NA-VCG5, and NA-VCG6 (Aqeel et al. 2008; Nitzan et al. 2006) for North American, Eu/I-VCG5 (Shcolnick et al. 2007) for Europe/Israel, and AUS-VCG4 for Australian populations (Ben-Daniel et al. 2010) were the most aggressive groups. VCG/AFLP2 and VCG/AFLP4/5 were found to be the most important globally distributed group with high genetic similarity between them. Durable resistance selected against any of these groups could mean that cultivars developed with resistance in one region would also be resistant to black dot in other regions thereby providing disease control for *C. coccodes* worldwide. Field resistance to stem colonization (Nitzan et al. 2009) and partial resistance to black dot in *Solanum tuberosum* group *Andigena* was identified on three genotypes (Nitzan et al. 2010). An isolate belonging to NA-VCG2 (R113) was used to evaluate black dot (Nitzan et al. 2010). In our study, both VCG/AFLP2 and VCG/AFLP4/5 had high genetic similarity (I=93 %), based on that, durable resistance selected against VCG/AFLP2 could be effective also for VCG/AFLP4/5 since these two VCG's form the largest groups globally.

In conclusion, by using AFLP analysis, *C. coccodes* populations from different geographic origin had clustered together in five VCG/AFLP groups (1, 2, 3, 4/5, and 6/7). VCG/AFLP 6/7 was very distinctive from the other VCG/AFLP's. VCG/AFLP4/5 was the most common VCG globally, followed by VCG/AFLP2. Furthermore, we reject the hypothesis that *C. coccodes* global populations are genetically different, however, it appears geographic isolation caused these populations to differentiate. These findings will assist in choosing the isolates for genetic resistance assessment and assuming standard worldwide assessment parameters for *C. coccodes* can be created, the development of stable resistance to black dot may be possible in future breeding studies.

Acknowledgments The authors thank Curt Doetkott for his assistance in statistical analysis. The authors also thank Sujan Mamidi for assistance and constructive comments on the manuscript. The authors express their appreciation to the Minnesota Area II Potato Growers Association and to the North Dakota State University for funding this research. The authors also extend their appreciation to N. Nitzan, H. Dilliard, D. Johnson, A. Lees and J. Miller for sharing isolates of *C. coccodes* from their culture collection. The authors thank J. Pasche and I. Mallik for their technical support.

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