Genetic Variability of *Phytophthora cinnamomi* Rands in Michoacan, Mexico

Yisa María Ochoa-Fuentes, Universidad Autónoma de Aguascalientes, Centro de Ciencias Agropecuarias, Departamento de Fitotecnia, Posta Zootécnica, Av. Universidad No. 940, Col. Ciudad Universitaria, Jesús María, Aguascalientes, México CP 20900; **Octavio Martínez-de la Vega**, **Víctor Olalde-Portugal**, Centro de Investigación y Estudios Avanzados, Lab. de Genética Cuantitativa y Lab. de Bioquímica Ecológica, Unidad Irapuato, Apdo. Postal 629, km 9.6 Libramiento Norte Irapuato-León, Irapuato, Guanajuato, México CP 36500; **Ernesto Cerna-Chávez, Jerónimo Landeros-Flores, Francisco Daniel Hernández-Castillo** y **Alberto Flores-Olivas,** Universidad Autónoma Agraria Antonio Narro, Depto. de Parasitología, Apdo. Postal 342, km 7.5 Carr. a Zacatecas, Buenavista, Saltillo, Coahuila, México CP 25315. Correspondencia: yisa8a@yahoo.com

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Abstract. Thirty eight isolates of *Phytophthora cinnamomi* from five counties in Michoacan, Mexico, were genetically analyzed using amplified fragment length polymorphisms (AFLP) to determine the genetic variability and the existing relationships among the avocado isolates. The amplifications showed 296 bands. A genetic dissimilarity matrix was obtained in this trial by means of the multiple coupling coefficient and the hierarchical grouping method, also called non-weighted average method. The resulting dendogram showed two levels of association. The isolates were classified within two main groups associated with the geographical location from which they were obtained. The analysis and determination of the confidence levels demonstrated that these geographical groups are strong.

Additional keywords: AFLP, *Persea americana*, avocado, tristeza.

Resumen. Treinta y ocho aislados de *Phytophthora cinnamomi* de cinco municipios del estado de Michoacán, México, se analizaron genéticamente usando polimorfismos en la longitud de fragmentos amplificados (AFLP), con el objetivo de determinar la diversidad genética y la relación entre los aislados obtenidos de aguacate. Las amplificaciones mostraron 296 bandas; el análisis consistió en la obtención de la matriz de disimilaridades genéticas utilizando el coeficiente de apareamiento múltiple y el método de agrupamiento jerárquico o método del promedio no ponderado. El dendograma obtenido mostró dos niveles de asociación. Los aislados se clasificaron dentro de dos grupos principales asociados con la localización geográfica de la cual se obtuvieron. El análisis y la determinación de los niveles de confianza muestran que estas agrupaciones geográficas son fuertes.

Palabras clave adicionales: AFLP, *Persea americana*, aguacate, tristeza.

Phytophthora cinnamomi Rands causes tristeza, one of the most devastating diseases of avocado (*Persea americana* Mill.) fruit tree (Zentmyer, 1980). This pathogen has two sexual compatibility groups: A1 and A2 (Alexopoulos *et al*., 1996; Erwin and Ribeiro, 1996). Sexual condition is a potential source of intra-specific variability in heterothallic species (Boccas and Zentmyer, 1976). An alarming number of trees are currently suffering from *P. cinnamomi* in Michoacan, Mexico. Conservative data establishes that 5% of this fruit crop has been already damaged by the disease (Téliz, 2000), and this percentage is expected to increase. Amplified fragment length polymorphisms (AFLP) depend on the enzyme recognition of the restriction sites in the DNA, the relationships among the number of initiating oligonucleotides selective nucleotides and the genome's complexity (Lin and Kuo, 1995). The AFLP technique has been used for genetic characterization of a variety of organisms including bacteria, plants, and nematodes (Becker *et al*., 1995; Folkertsma *et al*., 1996; Huyys *et al*., 1996). Recent applications include genetic characterization of plant pathogenic fungi. The AFLP method allowed assessment of pathogen diversity and species determination

(Farr *et al*., 2000; O Neill *et al*., 1997; van der Lee *et al*., 1997). Besides their use in plant breeding, genome mapping, and in populations´ biology (Breyne *et al*., 1997), strength stems from the simultaneously observed loci and their consistency. At present, the genetic diversity of *P. cinnamomi* in Michoacan, Mexico, has not yet been determined. Molecular trials such as AFLP are needed to clarify that question and to design control strategies. The purpose of this essay was to determine the genetic variability and the existing relationships among thirty eight isolates of *P. cinnamomi* obtained from the main avocado producing areas in Michoacan, Mexico using AFLP.

MATERIALS AND METHODS

Thirty eight root and soil samples from avocado trees showing symptoms of *P. cinnamomi* were obtained from the municipalities of Tancitaro, Periban, Salvador Escalante, San Juan Nuevo, and Uruapan in the state of Michoacan, Mexico. V-8-agar cultural medium was used to isolate and purify the pathogen from root samples. The medium was prepared by mixing 2 g of $CaCO_3$ with 200 mL of V-8 juice and centrifuged at 3,500 rpm during 20 min; 200 mL of the supernatant were diluted with 800 mL of sterile distilled water, 16 g of agar (Zentmyer *et al*., 1976), and 20 g of anhydrous dextrose were added to the mix, before leaving it at 24°C ± 2 in the dark for 7 days. The apple fruit method was used to isolate the pathogen from soil samples. Three 3 cm deep holes were punched in an apple to put the soil samples inside. A cotton ball with sterile distilled water covered the hole to keep it wet. The apple peel was placed on top using an adhesive tape. After inoculation, the fruit was placed in plastic bags inside a wet chamber during 48 h at 24ºC. Samples were checked every two days. Positive samples developed chestnut stains around the holes and they were used in the isolation and purification processes made on V-8-agar (Campbell, 1949; Róndon *et al*., 1988).

***P. cinnamomi* identification**. The preparations obtained from the cultural medium were studied under the microscope and they were identified using morphological criteria (Erwin and Ribeiro, 1996; Huberli *et al*., 1997). Then, the material was multiplied using soil extract inoculations that enabled to obtain a larger number of sporangia (Ayers and Zentmyer, 1971).

DNA extraction. DNA extraction was made by scraping mycelia of *P. cinnamomi* developed in the clarified V-8 agar culture medium. DNA was extracted using Doyle and Doyle's method (1990), as modified by Vazquez with sodium dodecyl sulfate (SDS; Promega Inc); 0.2 g of the mycelia were macerated with a ground rod before being re-suspended in 500 µL of extraction buffer (Tris-HCl, pH 8.0 100 mM, EDTA pH 8.5 50 mM, NaCl 50 mM and SDS 2%). The suspension was placed into the vortex for 30 seg before being left on ice during 15 min; 500 µL of isoamyl chloroform alcohol 24:1 were added, stirred in the vortex and centrifuged at 12,000 rpm during 15 min; the supernatant was recovered in a new eppendorf adding the same volume of isopropanol. The mix rested on ice for 15 min before being centrifuged at 12,000 rpm during 10 min. The supernatant was discarded to recover the tablet. The tablet was re-suspended in 50 µL of sterile de-ionized water, adding 10 µL of RNasa at 37°C for 1 h. Finally, the right DNA concentration for the PCR reaction was determined, using 100 ng/µL.

AFLP Essay. The protocol used in the essay is similar to the protocol reported by Vos *et al*. (1995), except for the use of markers with 700 and 800 nm fluorchromes in *Eco* initiators.

Digestion-ligation of DNA. Digestion was achieved with DNA samples at a concentration of 100 ng/µL. After checking their quality in agarose gels at 1%, 1 µL of diluted DNA from each sample was placed on a PCR plate following a previously determined arrangement. Eight µL of sterile distilled water and 3.5 µL of the mix (1 µL of DNA at 100 ng/µL, 0.5 µL of *Eco* RI at 10 U/µL, 0.5 µL of *Tru* 91 at 10 U/µL, 2.5 µL of Buffer RL) were added to every reaction. To achieve digestion, the mix was incubated at 37ºC for 2 h and 15 min; and at 70ºC to inactivate the enzyme, using a thermocycler GeneAmp® PCR Systems 9700 (Applied Biosystems). The mix was stored at 4°C. Afterwards, 12.5 µL of the ligation mix were added to every reaction [1.0 µL of Adapter *Eco* RI/ *Eco* RI (5 pmol), 1.0 µL of Adapter *Mse* I/*Mse* I (50 pmol), 1.0 µL of ATP (10 mM) pH 7, 9.0 µL of Buffer RL (5X), and 0.5 µL of T4 DNA ligase] and they were incubated at 20ºC for 2 h, inside the thermocycler. When the ligation process was completed, a dilution of 1:60 (2 µL DNA + 118 water) was prepared and stored at -20ºC.

First amplification. Primers 5-GACTGCGTACCAATTC/A-1 (*Eco* RI) and 5-GATGAGTCCTGAGTAA/C-1 (MseI) were used in the first amplification, 2.5 µL of digested-bonded DNA (diluted 1:60) were placed in the PCR plate, along with 23 µL of the following mix: the amplification reaction 1.5 µL of E + A (50 ng/µL), 1.5 µL of M + C (50 ng/µL), 2.5 µL of Buffer 10X (Mg), 0.5 µL of Taq DNA polymerase (5U/µL), 2.5 µL of DNTPs (10 mM) and 14.5 µL of sterile water. A thermocycler was used in the amplification process, according to the following schedule: 5 min cycle at 95ºC; 0.5 min at 94ºC; 1 min at 56ºC, and 1 min at 72ºC; 20 5 min cycles at 95ºC; 0.5 min at 94ºC; 1 min at 56ºC; 1 min at 72ºC, and at 4ºC. A 1:60 dilution (2 µL DNA + 118 µL of water). The pre-amplification reaction was prepared and stored at 4ºC and at -20ºC.

Second amplification. The selective amplifications of this essay used 4 combinations of primers (IRDye Flourecent AFLP®)(E-ACA700/M-CTC, E-ACC700/M-CAT, E-ACT800/ M-CAT, and E-ACG800/M-CTT. A blend for every combination was prepared [2.0 µL of DNA template first amplification (Dil 1:60), 2.0 µL of M + NNN (50 ng/µL), 0.5 µL E + NNN 700 (50 ng/µL), 0.5 µL of E + NNN 800 (50 ng/µL), 1.2 µL of Buffer 10X, 0.1 µL Taq DNA polymerase (5U/µL), and 4.8 µL of sterile water]. The amplification reaction took place inside the thermocycler according to the following schedule: 5 min at 94ºC; 12 cycles of 0.5 min at 94ºC; 0.5 min at 65ºC, and 1.0 min a 72ºC, lowering the alignment temperature by 0.7ºC in each cycle; 23 cycles of 0.5 min at 94ºC; 0.5 min at 56ºC; 1 min

at 72ºC and 4ºC. The selective amplification products were submitted to a polyacrylamide sequencing gel and analyzed on a LICOR sequencer. Using the AFLP automatic sequencer (LI-COR SAGA MX software release 2.1). 20 mL of buffer KBplus at 6.5% (Acryl amide at 40%, Urea 8 M and TBE) with 150 µL of an ammonium per-sulfate solution (APS) at 10% and 15 µL TEMED (N,N,N,N,- Tetra-methyl ethyl diamine), Polyacrylamide was poured between the crystal plates with a syringe to avoid bubble formation. A shark-like comb was inserted before clamping the pressure plates. Polymerization lasted 1 h. The gel was treated for 20 min inside the sequencer before loading the samples, using buffer TBE 0.8 X. After that time, 0.4 µL of denatured DNA (5 min at 94ºC) were loaded already mixed with a blue dye buffer and a load of 6 X. A molecular marker of 50-700 bases was used. The Electrophoresis process lasted 2 h at the equipment´s specified operating conditions. The gel images were analyzed using software LI-COR SAGA MX release 2.1 for gel reading. The molecular weight of bands was obtained to use as a reference for each marker when reading the polymorphic bands in each lane.

Data analysis. Windows S-plus professional software was used in the data analysis. A genetic dissimilarity matrix was obtained using the multiple coupling coefficient (Sneath and Sokal, 1973), also known as the Sckroch´s coefficient (Skroch *et al*., 1992), followed by the hierarchical grouping method, UPGMA or non-weighted average method to produce a dendogram (Everit, 1993; Sneath and Sokal, 1973). Felsenstein's confidence coefficients (Felsenstein, 1985) were obtained and used in the dendogram nodes.

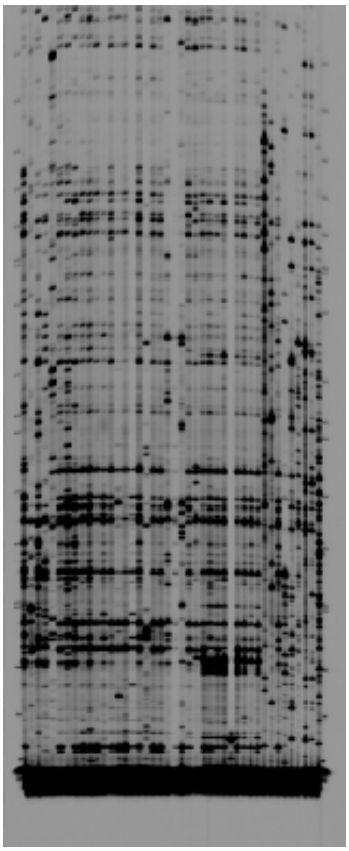

Fig. 1. Band pattern obtained by amplified fragment length polymorphisms using a combination of initiator E-ACA700/ M-CTC and a 50-700 bp marker.

RESULTS AND DISCUSSION

Thirty eight *P. cinnamomi* isolates were analyzed with AFLP, using four combinations of initiators. The analysis revealed 296 bands in total, ranging from 55 to 639 bp. Figure 1 shows the profile of amplifications obtained with initiator E-ACA700/ M-CTC. Bands were classified either as monomorphic or as polymorphic; E-ACA700/M-CTC, E-ACC700/M-CAT, E-ACT800/M-CAT, and E-ACG800/M-CTT produced 60.8, 72, 66, and 34% of the polymorphic bands, out of a total of 130, 67, 46, and 53 amplified bands. The 296 bands from isolates were analyzed using Windows S-plus professional software. The data obtained from AFLP were used to produce a genetic dissimilarity matrix, using the multiple coupling coefficient (Sneath and Sokal, 1973), also called Sckroch's coefficient (Skroch *et al*., 1992), followed by the hierarchical grouping method, UPGMA or non-weighted average method, to produce a dendogram (Sneath and Sokal, 1973). The dendogram showed two levels of association. Isolates were classified in two main groups associated with the geographical locations from which they were obtained. The analysis and determination of the confidence levels showed that these geographical groupings are strong. In order to obtain Felsenstein's confidence coefficients (Felsenstein, 1985), for the dendogram nodes, a total of B = 1000 bootstrap replications. The reported dendogram shows the percentage of times that a node is repeated in the B = 1000 replications. When comparing the population of *P. cinnamomi* in the five municipalities of Michoacan, Mexico, it was observed the prevalence of geographical distribution in the relationship of isolates. Based on the dendogram analysis and the geographical origin of the isolates (Fig. 2), group A contains 100% of the isolates from Salvador Escalante, 80% of the isolates from Periban, 50% of the isolates from Tancitaro, 77.7 % of the isolates from San Juan, and 62.5% of the isolates from Uruapan. Group B contains 22.2% of the isolates from San Juan Nuevo, 37.5% of the isolates from Uruapan, 50% of the isolates from Tancitaro, and 20% of the isolates from Periban. Ten isolates from the sampling sites in group A come from similar climate conditions: an average altitude of 1950 m above the sea level, with summer rainfall (average 900 mm), temperatures ranging from 7 to 38°C, andosol humic-ochric slightly drained soil, used mainly in forestry and in agriculture to a lesser extent, with a predominance of coniferous woods with fir-spruce trees (oyamel) and junipers, as well as a mix of oak-pine trees (ASEEAM, 1998). The information obtained by AFLPs genetic fingerprint, depicted in the dendogram

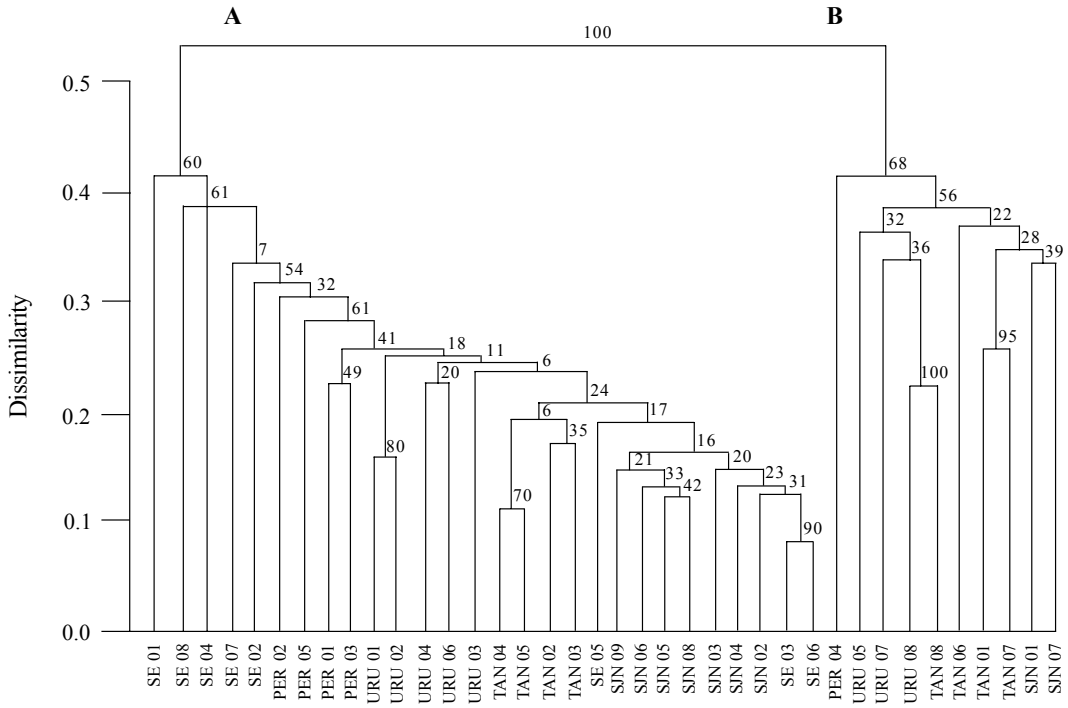

Fig. 2. Amplified fragment length polymorphisms'dendogram of *Phytophthora cinnamomi* from Michoacan, Mexico. Two main groups are shown: A and B. Municipalities: SE = Salvador Escalante; PER = Periban; URU = Uruapan; TAN = Tancitaro; SJN = San Juan Nuevo.

shows no visible level of clonicity between A and B groups. The minimum level of dissimilarity between the isolates was 0.1, out of which 10% corresponded to differences in band patterns. These results discard the possibility of having clone pairs in the isolate collection. According to the results, *P. cinnamomi* estimated genetic distances coincide with the reports of other authors (Ivors *et al*., 2004) regarding other species of the same genus. This pathogen has a high level of genetic variability closely correlated to the geographical source of the isolates. The two dendogram groups indicate a source of genetic variability resulting from sexual and asexual reproduction of the pathogen; since *P. cinnamomi* is an important root pathogen for a large number of hosts mainly found in forestry regions all over the world (Podger, 1972; Shea *et al*., 1983; Thorn and Zentmyer, 1954; Zak and Campell, 1958; Zentmyer, 1980). Sexual reproduction in *Phytophthora* species has had a significant influence in the population's genotypic diversity of these pathogens. For instance, the introduction of both compatibility groups of *Phytophthora infestans* (Mont.) de Bary within Europe, increased the genotypic diversity of this pathogen (Drenth *et al*., 1994). In that regard, *P. cinnamomi* isolates from South Africa presented significant genetic differences (Linde *et al*., 1997, 1999). In populations of *P. infestans* around the world, genetic variation might not be totally attributed to sexual reproduction (Goodwin *et al*., 1994; Xu *et al*., 1999). Genetic variability derived from mitotic recombination may account for phenotypic variability which suggests the occurrence of *P. cinnamomi* clones in the world (Oudermans and Coffey, 1991). The hypothesis regarding the outstanding variation in Australian populations of *P. cinnamomi*, due to sexual and asexual reproduction has already been demonstrated by Dudzinski *et al*. (1993). The impact of genotypic and phenotypic variation of *P. cinnamomi* populations in the world is not yet clearly known; however, asexual reproduction is an important variation mechanism in the pathogen populations. Huberli *et al*. (1997, 2001) showed a substantial phenotypic variation in a clonal line of *P. cinnamomi*, in populations that were 70 km apart, this was the first study conducted on *P. cinnamomi* with a large number of isolates, in which genotypes and phenotypes varied greatly in populations that were close to each other. The information obtained in our research shows genetic variability of *P. cinnamomi* in avocado from the growing region of Michoacan, Mexico; an aspect that should be taken into account for future disease management investigations.

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