Isolation and Molecular Identification of *Curvularia lunata/Cochliobolus lunatus* Causal Agent of Leaf Spot Disease of Cocoa

Cuervo-Parra, J. A.[1]*, Romero-Cortes, T[1], Ortiz, Y. G.[1] and Ramírez-Lepe, M.[2]
[1]Universidad Autónoma del Estado de Hidalgo, Hidalgo, México. Carretera Pachuca Tulancingo km 4.5, Mineral de la Reforma. Hidalgo. México. C.P. 42090 Tel (771) 7172000 ext 4009.
[2] Instituto Tecnológico de Veracruz . Unidad de Investigación y Desarrollo en Alimentos. Av. M.A. de Quevedo No. 2779 Col. Formando Hogar C.P. 91897 Veracruz, Ver. Fax: (229) 934 57 01.
*E. Mail: jalioscha@gmail.com

Introduction

Cocoa (*Theobroma cacao* L.) is a crop with great commercial value in many tropical regions worldwide, whose fruit is processed in the industry in many products, cosmetics and medicine industry. However, plantations generate a large volume of waste that generally remain scattered into the plantation and cause the phytopathogen propagation, affecting the cocoa plant and plays an important role in the destruction of their fruits. The most important are the frosty pod rot caused by *Moniliophthora roreri* (1), witches' broom caused by *Moniliophthora perniciosa* (2), black pod caused by *Phytophthora* spp. (3), and leaf spot caused by *Curvularia lunata*/*Cochliobolus lunatus* (4). *Curvularia* is a hyphomycete fungus which is a facultative pathogen of many plant species. Most *Curvularia* are found in tropical regions, though a few are found in temperate zones. The teleomorph state of the type species *Curvularia lunata* is *Cochliobolus lunatus*. In Mexico, few works has been focused on the molecular identification of fungi cocoa diseases (i.e. *M. roreri*). In particular in the Chontalpa region, located in the center-west of the Tabasco state, Mexico, the fungi have been the most aggressive. The identification of phytopathogenic strains is important for developing strategies for its prevention and control. Molecular techniques such amplification of DNA conserved sequences by Polymerase Chain Reaction (PCR) have proven to be useful in studies of genetic diversity, life cycle, ecology, phylogeny and identification of microorganisms. The technique known as PCR-ITS (Internal Transcribed Spacer-PCR) allows replicate *in vitro* thousands of times, specific sequences of the inner region of ribosomal genes of fungal species. Therefore the sequences of the nucleotides are compared to others from GeneBank, allowing its taxonomic identification. The genes are amplified and compared the ribosomal DNA (18S, 5.8S and 28S), which are highly preserved (5). The objective of this study, was the molecular characterization of five strains of fungi, by means of the sequenciation of the ITS I/5.8s/ITS II region of the DNAr of all fungus previously identified as *Curvularia lunata*, *C. hawaiiensis* and *Bipolaris tetramera* by morphological tecniques, obtained through diseased fruits of cacao plants from the Chontalpa area of the state of Tabasco, Mexico.

Materials and Methods

Fungal strains

The strains HT-ITV03, HT-ITV40, HT-ITV45, HT-ITV46 and HT-ITV48 used in this study were isolated from diseased fruits of cocoa trees collected during October 2007 and November 2009, respectively. The samples of diseased fruits were taken from three commercial plantations located in the municipality of Huimanguillo, Tabasco. For isolating fungi, fragments of diseased fruits were placed in a moist chamber and incubated at 25ºC for 3 days in darkness. After incubation, individual fungi were recovered as they grew out of the diseased tissue and then subcultured on potato dextrose agar (PDA) at room temperature for 5 days to obtain pure cultures. All fungal strains were maintained on PDA at 4°C and as a suspension of spores at -74ºC. In addition, the strains were subcultured every month on PDA medium.

DNA extraction, PCR and sequencing

Extraction of DNA from *Cochliobolus lunatus*, *C. hawaiiensis* and *B. tetramera* strains were performed as described previously Kurzatkowski *et al*. in 1996 (6). A region of nuclear DNA, containing the ITS I,

5.8S, and ITS II sequence, for each strain were amplified by PCR using the primers ITS1 (5'-tccgtaggtgaacctgcgg-3') and ITS4 (5'-tcctccgcttattgatatgc-3') (7). PCR amplifications were performed in a total volume of 50µl reaction, which contained: 10 µL of 5x Colorless buffer, 200 µM dNTPSs, 0.2 µM of each primer, 2.5 units of Taq DNA polymerase, and 10–50 ng of template DNA. The PCR reactions were placed in a Bio-Rad thermal cycler (Bio-Rad Laboratories, Hercules, California, USA) under the following parameters: 5 min initial denaturation at 95ºC, followed by 30 cycles of 1 min denaturation at 95ºC, 1 min primer annealing at 57ºC, 1 min extension at 72ºC and a final extension period of 12 min at 72ºC. The resulting products were purified with the Geneclean® II kit (Bio101 Inc., California, USA) according to the manufacturer's protocol. DNA samples were sequenced by the Instituto de Biotecnología, Cuernavaca, Mor., Mexico.

DNA sequence analysis

DNA sequences from each strain were assembled by use of Chromas 1.45 (School of Health science, Griffi th University, Gold Coast Campus, Southport, Queensland, Australia), and then aligned using the ClustalX inference package (8). The sequences were subjected to the basic local alignment search tool (BLAST) available at http://blast.ncbi.nlm.nih.gov/Blast.cgi against sequences available in GenBank. Phylogenetic analysis of the aligned sequences was done by Neighbour-Joining analysis with 1000 bootstrap replicates and was supported by bootstrap method. Unrooted phylogenetic tree was constructed with help from the PhyloDraw program 0.8 (9).

Results and Discussion

Isolation of strains

Five random samples of cocoa fruits with symptoms of disease of each of the three plantations were collected. One hundred and twenty eight strains were isolated of which three were identified by their morphological characteristics as *C. lunatus* and one as *C. hawaiiensis* and *B. tetramera*, respectively. The isolated species of the *Cochliobolus* genus (*C. hawaiiensis* and *C. lunatus*), were already reported by other authors as pathogen of plants like corn (10), barley (11), oat, sorghum, banana, pineapple (12) and many fescue (13). These fungi cause the leaf spot and the putrefaction of the vascular tissue and fruits reasons because these pathogen produce cocoa losses. This genus of fungi are the teleomorph state of many species of fungi of the genus *Bipolaris* (14) and *Curvularia* (15) genus that were reported as pathogen or the cocoa crop by others authors (4). By other hand, the anamorph state of *C. hawaiiensis* was reported and isolates in Tabasco state by others authors (16) represented the first report for the isolation of the teleomorphic state in Mexico.

Molecular identification and phylogenetic analysis

For the three strains of *C. lunatus* and the individual isolates of *C. hawaiiensis* and *B. tetramera*, were obtained the entire sequences of the ITS I/5.8s/ITS II region, together with the ends of the 18S and 28S subunits. The BLAST search confirmed the morphological identification of the sequences of *C. lunatus*, *C. hawaiiensis* and *B. tetramera*. The sequences examined in this study for the ITS I/5.8s/ITS II region, for the strains of *C. lunatus*, *C. hawaiiensis* and *B. tetramera* and other related sequences from GenBank had a length of between 506 to 629 bp for the species examined in this study of *Cochliobolus* spp. and *Bipolaris* sp. and the length of the consensus region for alignment of all sequences was 697 bp (Table 1). For the analysis of 5.8S subunit was observed to be highly conserved between all species of *Cochliobolus* and *Bipolaris*, with change limited to the variations of a few bases in the sequences of *C. lunatus*. On the other hand, in the 18S and 28S subunits, was observed much variability, which was limited to point mutations, insertions or deletions, predominantly among isolates of *C. hawaiiensis*, *B. tetramera* and *B. spicifera*. Much variation was observed in terms of deletions, insertions and base substitutions within the ITS regions, especially in the ITS II, although many conserved regions were present in the ITS region I and ITS II. In general the length of the sequences of the ITS I and ITS II regions was highly conserved in all species of *Cochliobolus* and *Bipolaris*, with limited variations to the change of a few bases.

Phylogenetic analysis of sequences of strains of *C. lunatus*, *C. hawaiiensis*, *B. tetramera* and others 24 sequences of *Cochliobolus* and *Bipolaris* related of GenBank, generated a tree by the Neighbor Joining method that grouped isolated from the strains HT-ITV03, HT-ITV40 and HT-ITV45, in one same clade and were aligned along a brother clade formed by sequences of the strains IP 1417.82, IP 2328.95, IP 613.60 and AHB-1 of *C. lunatus* (DQ836799, DQ836800, DQ836798 and GQ280375) the first three isolated in France by Desnos-Ollivier *et al*., in the year 2006 (15) and the fourth isolated in Mexico by Tapia-Tussell *et al*., in the year 2009 (17), supported by a bootstrap value of 929. On the other hand, for sequences of *C. hawaiiensis* and *B. tetramera* isolated in this study, we observed that lined up in different clades. The HT-ITV46 strain, was aligned with the sequence of strain Alcorn 7612(b)-6 of *C. hawaiiensis* (AF071324) isolated in Canada by Berbee *et al*. in the year 1998 (18) and HT-ITV48 strain was aligned with the strain BMP 51-31-01 of *B. tetramera* (AF229477) isolated in the United States by Pryor and Gilbertson in the year 2000 (19).

Table 1. *Bipolaris* and *Cochliobolus* genus, ITS I/5.8s/ITS II region and 18S and 28S genes for the aligned sequences

GenBank Access number	Gene 18S length (bp)	ITS I length (bp)	Gene 5.8S length (bp)	ITS II length (bp)	Gene 28S length (bp)	Total length
HT-ITV45	44	162	158	162	103	629
HT-ITV40	46	163	159	164	74	606
HT-ITV03	55	162	156	162	78	613
DQ836799	55	160	158	163	59	595
DQ836800	55	162	158	164	59	598
GQ280375	21	161	158	165	50	555
DQ836798	55	162	158	165	59	599
AY253918	39	161	158	161	42	561
EF503562	45	161	158	161	38	563
AJ303084	32	161	158	161	40	552
AF229477	11	161	158	161	41	532
HT-ITV48	8	161	158	161	47	535
HT-ITV46	30	161	158	161	33	543
AF071324	11	161	158	159	17	506
FR717533	30	161	158	161	65	575
AF158106	27	161	158	159	21	526
GU480767	32	165	158	161	60	576
EF540752	29	161	158	161	60	569
AF071322	11	161	158	159	17	506
AJ853762	8	161	158	161	40	528
HM195268	31	161	158	162	60	572
FJ040180	30	161	158	162	58	569
AY923860	30	161	158	162	59	570
HM195265	31	161	158	162	60	572
HM195264	30	161	158	162	60	571
HM195263	22	161	158	162	61	564
HQ248191	0	150	158	164	60	532
GU183125	16	161	158	161	114	610
HT-ITV45	0	114	158	164	114	550
Length range :	0 to 55	114 to 165	156 to 159	159 to 165	17 to 114	506 to 629
Consensus sequence:	1-55 bp	56-227 bp	228-385 bp	386-555 bp	556-697 bp	1-697 bp

This result, confirms that the strains identified as *C. lunatus*, *C. hawaiiensis* and *B. tetramera* may belong to different specimens of these species due to bootstrap values that support these alignments (Figure 1). Also, with the phylogenetic analysis performed, was corroborated the findings by Worapattamasri *et al*. (20) since it was found that *C. hawaiiensis* is a heterothallic fungus and that *B. hawaiiensis* is its anamorphic state. On the other hand, Weikert-Oliveira *et al*. in 2002 (21) reported that *Bipolaris* species are genetically distinguishable and showed that the teleomorph of the analyzed species have high genetic similarity in their electrophoretic profiles when were compared with their respective anamorphs. According to Burdon and Silk (22), a major source of genetic variation in phytopathogenic fungi, are

mutation and recombination. Furthermore, within a species, genes flow between populations and supplementing this process as propagules which diffuse from one epidemiological area to another and from one deme to the next.

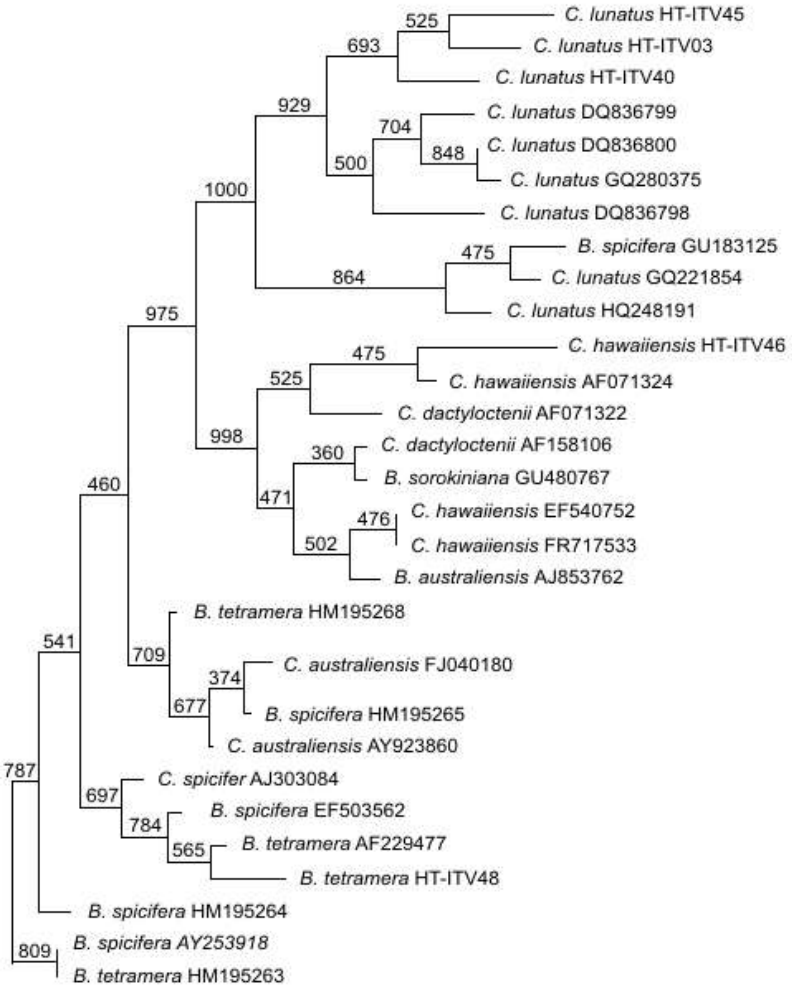

Figure 1. Neighbour-Joining tree derived from fungal ITS sequence data. Numbers on nodes are bootstrap values, i.e. The frequency (%) with which a cluster appeared in a bootstrap test of 1000 runs with Kimura 2-parameters. HT-ITV03, HT-ITV40, HT-ITV45, HT-ITV46 and HT-ITV48 isolated from *T. cacao*.

References

1) Cuervo-Parra, J. A., Sánchez-López, V., Ramirez-Suero, M., and Ramírez-Lepe, M. Morphological and Molecular Characterization of *Moniliophthora roreri* Causal Agent of Frosty Pod Rot of Cocoa Tree in Tabasco, Mexico. 2011. Plant Pathology. 10(3): 122-127. ISSN: 1812-5387. DOI: 10.3923/ppj.2011.122.127

2) Kilaru1, A., Bailey, B. A., and Hasenstein, K. H. *Moniliophthora perniciosa* produces hormones and alters endogenous auxinand salicylic acid in infected cocoa leaves. 2007. Federation of European Microbiological Societies. 274(2007): 238-244. DOI:10.1111/j.1574-6968.2007.00837.x

3) Djocgoue, P. F., Boudjeko, T., Nankeu, D. J., Efombagn, M. I. B., Nyasse, S., and Omokolo, D. N. Comparative assessment of the resistance of cocoa (*Theobroma cacao* L.) progenies from SNK10 x SNK413; ICS84 x ICS95 to *Phytophthora megakarya* in Cameroon by measuring size of necrotic lesion along the midrib. 2006. Plant Pathology. 5: 329-335.

4) Urdaneta, G. L., and Delgado, A. A. Identificación de la microciota del filoplano del cacaotero (*Theobroma cacao* L.), en el municipio Carraciolo Parra Olmedo, estado Mérida, Venezuela. 2007. Revista de la Facultad de Agronomia (LUZ). 24:47-68.
5) Foster, D. M., Kozak, K. R., Loftus, M. G., Stevens, J. J., and Ross, I. K. The polymerase chain reaction and its application to filamentous fungi. 1993. Mycological Research. 97:769-781.
6) Kurzatkowski, W., Törrönen, A., Filipek, J., Mach, R., Herzog, P., Sowka, S., and Kubicek, C.P. Glucose-Induced Secretion of *Trichoderma reesei* Xylanases. 1996. Applied And Environmental Microbiology. 62(8): 2859–2865.
7) Foster, D. M., Kozak, K. R., Loftus, M. G., Stevens, J. J., and Ross, I. K. The polymerase chain reaction and its application to filamentous fungi. 1993. Mycological Research. 97:769-781.
8) Larkin M.A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H., Valentin F., Wallace I.M., Wilm A., Lopez R., Thompson J.D., Gibson T.J., and Higgins D.G. ClustalW and ClustalX version 2. 2007. Bioinformatics. 23(21): 2947-2948.
9) PhyloDraw versión 0.8. 1999. Graphics Application Lab, Pusan National University. Consultado el 30 de julio del 2008. http://search.wareseeker.com/phylodraw-version-0.8/.
10) Tonukari, J. N., Scott-Craig, J. S., and Waltonb, D. J. The *Cochliobolus carbonum SNF1* Gene Is Required for Cell Wall–Degrading Enzyme Expression and Virulence on Maize. 2000. The Plant Cell. (12): 237-247.
11) Clay, P. R., Bergmann, W. C., and Fuller, S. M. Isolation and Characterization of an Endopolygalacturonase from *Cochliobolus sativus* and a Cytological Study of Fungal Penetration of Barley. 1997. Phytopathology. Vol. 87 (11): 1148-1159.
12) Sivanesan, A. *Cochliobolus verruculosus*, CMI Descriptions of fungi and bacteria. 1990. Mycopathology. 111, (2): 119-120.
13) Smith, M. A., Watson, Jr., C. E., Linscombe, S. D., and Trevathan, L. E. Selection for resistance to *Cochliobolus sativus* in tall fescue (*Festuca arundinacea* Schreb.). 1986. 72 (6): 807-810.
14) Nicolet, J., Gindrat, D., and Bille, J. GUIDELINES Classification of organisms Fungi. 2004. Swiss Agency for the Environment, Forests and Landscape SAEFL, Berne. pp. 115.
15) Desnos-Ollivier, M., Bretagne, S., Dromer, F., Lortholary, O., and Dannaoui, E. Molecular identification of Black-grain mycetoma agents. 2006. Journal of Clinical Microbiology. 44 (10): 3517-3523.
16) Mena-Portales, J., Mercado-Sierra, A., and Heredia, G. Especies de *Bipolaris* y *Curvularia* halladas sobre hojas de *Quercus* y *Liquidambar* en el estado de Veracruz, México. 1995. Revista Mexicana de Micología. 11: 109–121.
17) Tapia-Tussell, R., Solis, S., and Perez-Brito, D. Identification and molecular characterization of ligninolitic fungi from Yucatan, Mexico. 2009. GenBank data base. [Consulted 20-03-2010]. On line: http://www.ncbi.nlm.nih.gov/nuccore/gq280375
18) Berbee, M., Pirseyedi, M., and Hubbard, S. *Cochliobolus hawaiiensis* Direct submission. 1998. GenBank data base. [Consulted 20-03-2010]. On line: http://www.ncbi.nlm.nih.gov/nuccore/AF071324
19) Pryor, B. M., and Gilbertson, R. L. Molecular phylogenetic relationships amongst Alternaria species and related fungi based upon analysis of nuclear ITS and mt SSU rDNA sequences. 2000. Mycological Research. 104 (11), 1312-1321.
20) Worapattamasri, J., Ninsuwan, N., Chuenchit, S., and Petcharat, V. 2009. Anamorphs of *Cochliobolus* on disease plants in Southern Thailand. 2009. Journal of Agricultural Technology. (1): 143-155.
21) Weikert-Oliveira, R. C. B., Resende, M. A., Valerio, H. M., Caligiorne, R. B., and Pavia, E. Genetic variation among pathogens causing Helminthosporium diseases of rice, maize and wheat. 2002. *Fitopathol Brasil.* 27: 238-246.
22) Burdon, J. J., and Silk, J. Source and patterns of diversity in plant pathogenic fungi. 1997. Phytopathology. 87: 664-669.