Y/

Mycobiology 31(1): 19-22 (2003)

Molecular Analysis of Intraspecific Variations of the Indonesian *Cochliobolus heterostrophus*

Abdul Gafur*, Subli Mujim, Titik Nur Aeny, Budi Tjahjono[1] and Antonius Suwanto[2]

Department of Plant Protection, University of Lampung, Bandar Lampung 35145, Indonesia
[1]Department of Plant Pests and Diseases, Bogor Agricultural University, Bogor 16144, Indonesia
[2]Department of Biology, Bogor Agricultural University, Bogor 16144, Indonesia

The Korean Society of Mycology

Volume 31, Number 1, March 2003

Mycobiology 31(1): 19-22 (2003)

Molecular Analysis of Intraspecific Variations of the Indonesian *Cochliobolus heterostrophus*

Abdul Gafur*, Subli Mujim, Titik Nur Aeny, Budi Tjahjono[1] and Antonius Suwanto[2]

Department of Plant Protection, University of Lampung, Bandar Lampung 35145, Indonesia
[1]Department of Plant Pests and Diseases, Bogor Agricultural University, Bogor 16144, Indonesia
[2]Department of Biology, Bogor Agricultural University, Bogor 16144, Indonesia

(Received September 23, 2002)

The primary objective of the current research was to detect genetic variations within the Indonesian isolates of *Cochliobolus heterostrophus* collected from ecologically different places of the country at molecular level using PCR-RFLP analyses. The primer pair of NS3 and NS6 produced amplification fragment in all of the isolates tested. A single fragment of estimated 907 bp was observed in the PCR product pattern. RFLP analysis of the PCR product employing three restriction enzymes, *Hae*III, *Hha*I, and *Rsa*I, respectively, did not reveal intraspecific variations within the fungus. Similarly, nucleotide sequences of portion of small subunit of the ribosomal DNA gene of two of the isolates collected showed no appreciable differences, indicating the absence of genetic diversities among the isolates tested. A phylogenetic tree was constructed and the Indonesian *C. heterostrophus*, represented by SM-1 isolate, was found to be phylogenetically located near *C. sativus*, a closely related species.

KEYWORDS: *Bipolaris maydis*, *Cochliobolus heterostrophus*, Indonesian isolates, Intraspecific variations, PCR-RFLP

Cochliobolus heterostrophus (anamorph: *Bipolaris maydis*) is one of the most common plant pathogenic fungi on maize. Pathogenic races of the fungus cause an economically important southern corn leaf blight disease. The present research was initiated because the fungus had never been seriously explored in Indonesia and no information on genetic diversities at the molecular level of Indonesian isolates of the fungus was available. Accurate assessment of genetic diversity would enable further and deeper studies of genetic and other biological aspects of the fungus. This would surely benefit not only advancements of science and technology, but also practical aspects of evaluating biodiversity of ecosystem components and their ecological function for sustainable agricultural production.

In the past, intraspecific separation in *C. heterostrophus* was principally based on conidial morphology and toxin production (Bronson *et al*., 1990), and many races have been proposed (Alcorn, 1983; Sivanesan, 1987). Recent advances in DNA technology have allowed further assessment of genetic diversity in this species. A number of works employing different DNA-based methods have actually been initiated. Using contour-clamped homogenous electric field electrophoresis techniques of chromosomal DNA, Tanaka *et al.* (1992) found intraspecific variations within *C. heterostrophus* with four chromosomal size polymorphisms. Similarly, Nakada *et al.* (1994) detected three different types of Japanese isolates of the fungus using Restriction Fragment Length Polymorphism (RFLP) analysis.

In recent years, the Polymerase Chain Reaction (PCR)-RFLP analyses of ribosomal DNA (rDNA) have been developed and used as new tools for detection, identification, and phylogenetic studies of different fungal species (Landvik *et al*., 1998; Tae *et al*., 2002). For this purpose, rDNA is very useful because it is conserved genetically and has large copy numbers in genomes although internal transcribed spacer (ITS) regions including 5.8S of the gene have high genetic variations (Bruns *et al*., 1991). No studies, however, on the use of the PCR-RFLP technique to identify intraspecific variations of *C. heterostrophus* were reported. Therefore, efforts to employ the DNA-based method in this species were worth pursuing. The present research was the first attempt to differentiate intraspecific diversities in *C. heterostrophus* using PCR-RFLP analyses. The main objective of the current investigation was to detect genetic variations within Indonesian isolates of *C. heterostrophus* using the DNA-based technique.

Materials and Methods

Cultures and media. The research was conducted at the laboratory of Molecular Biology, Southeast Asian Regional Center for Tropical Biology (SEAMEO-BIOTROP), Bogor, Indonesia. The strains used in this study were different isolates of *C. heterostrophus* collected from ecologically different corn areas in Indonesia (Table 1) and laboratory strain HITO7711 (Tanaka *et al*., 1991). V-8 medium was used as the fungal growing medium for DNA extraction.

*Corresponding author <E-mail: gafur@uwalumni.com>

Table 1. Isolates of *Colchliobolus heterostrophus* used in this research and their origins

No.	Isolate	Origin
1.	BL-1	Singaraja, Bali
2.	JW-1	Banyuwangi, East Java
3.	JW-2	Jember, East Java
4.	JW-3	Bogor, West Java
5.	SL-1	Sungguminasa, South Sulawesi
6.	SL-2	Takalar, South Sulawesi
7.	SM-1	Tangganius, Lampung
8.	HITO7711	Laboratory strain (Tanaka *et al.*, 1991)

DNA extraction and PCR amplification. Total DNA was extracted using the methods developed by Nakada *et al.* (1994) and Yoder (1988) with some modifications (Gafur *et al.*, 1997; Gafur *et al.*, 1998). For DNA extraction, fungal isolates were cultured for two days in test tubes containing 3 m*l* of diluted V-8 medium broth at 25°C on a shaker. The cultured mycelia were recovered by centrifugation and lyophilyzation. Approximately 200 mg of mycelia were transferred to Eppendorf tubes, frozen in liquid nitrogen and finely powdered with a wooden applicator. The powder was suspended well in 600 μl of DNA extraction buffer (50 mM Tris-HCl pH 8.0, 125 mM EDTA pH 8.0, 50 mM NaCl, 0.1% (v/v) mercaptoethanol, and 2% (w/v) sodium N-lauryl sarcosinate). The suspension was deproteinized with equal volume of Tris-saturated phenol and equal volume of chloroform-isoamyl alcohol (24:1) before centrifugation at 15,000 rpm for 5 min. The aqueous phases were then transferred to new tubes, subjected to another chloroform-isoamyl alcohol (24:1) extraction, ethanol-precipitated (0.2 volume of 10 M sodium acetate and 2.5 volume of ethanol were added to the aqueous phases and stored at −80°C for more than 10 min) and again centrifuged at 15,000 rpm for 10 min. After being washed with 70% ethanol and air-dried, DNA was dissolved in TE buffer (10 mM Tris-HCl pH 8.0, and 1 mM EDTA pH 8.0).

Gene-specific primers used were derived from small subunit region of rDNA (Fig. 1) (Bruns *et al.*, 1991; White *et al.*, 1990). The primer pair of NS3 and NS6 (Table 2), based on conserved nucleotide sequences from the 18S region of rRNA genes from *Saccharomyces cerevisiae*, *Dictyostelium discoideum*, and *Stylonicha pustulata*, was employed in the present research to amplify the gene in the *C. heterostrophus*.

Table 2. Primers used in the present study

No.	Primer	Sequence
1.	NS3	GCAAGTCTGGTGCCAGCAGCC
2.	NS4	CTTCCGTCAATTCCTTTAAG
3.	NS6	GCATCACAGACCTGTTATTGCCTC

DNA amplifications were performed in standard 50 μl reactions containing 100 pmol of each primer, 1 μl of dioxynucleotide triphosphates (dNTPs), 5 μl of 10 × PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 1 unit of *Taq* DNA polymerase and approximately 32 ng of genomic DNA as a template. All reagents were preassembled in a bulk mix from which a 48-μl aliquot was dispensed into each of the 500-μl micro tubes before template DNA was added in 2-μl volumes. The mixtures were reacted on a GeneAmp PCR System 2400 (Applied Biosystems, USA) thermal cycler with the following thermal profile: initial denaturation at 95°C for three minutes followed by 25 repeated cycles of melting, annealing and extension of DNA at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 60 seconds, respectively. In the last cycle, the extension step was increased to 10 minutes to complete primer elongation. The expected 907 base pair (bp) PCR products were analyzed by agarose gel electrophoresis in 1 × Tris-acetate-EDTA (TAE) buffer (40 mM Tris-acetate pH 8.0, and 1 mM EDTA), stained with ethidium bromide and visualized under a UV transilluminator (Sambrook *et al.*, 1989). Gels were then documented using a Polaroid type 667 (Polaroid, USA) camera.

RFLP analysis. RFLP analysis of the PCR products was conducted according to the procedures explained by Nakamura *et al.* (1998). Amplified DNA fragments were partially purified as follows. Reaction mixture containing amplified DNA fragment was electrophoresed on a 1.5% (w/v) low temperature-melting agarose gel. A piece of gel containing the target fragment was cut off, transferred to an Eppendorf tube containing TE buffer. After heating the tube at 65°C, TE-saturated phenol of the same volume as gel plus TE buffer was added and the tube was shaken. After centrifugation at 14,000 rpm at 10 min, the aqueous phase was transferred to a new tube, an equal volume of chloroform was added and the tube was shaken. The

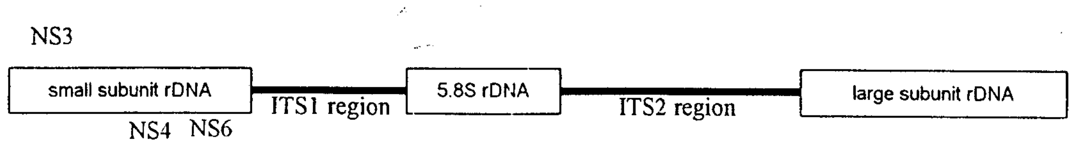

Fig. 1. Representation of the ribosomal DNA repeat, showing small subunit, internal transcribed spacer, 5.8S and large subunit regions. The primers used in the present study are derived from the small subunit region.

aqueous phase was again transferred to another new tube and 1/10 volume of 3 M sodium acetate and 7/10 volume of 2-propanol were added. After centrifugation, the precipitates were washed with 70% (v/v) ethanol, air-dried and dissolved in TE buffer. The purified fragments (approximately 50 ng) were digested with three different restriction enzymes, *Hae*III, *Hha*I, and *Rsa*I, overnight at 37°C, according to the manufacturersí instructions. The digested fragments were separated by 8% (w/v) polyacrylamide gel electrophoresis in TAE buffer, stained with ethidium bromide, visualized under a UV transilluminator (Sambrook *et al.*, 1989), and documented.

DNA sequencing. To confirm the results of the PCR-RFLP analyses and investigate phylogenetic position of the Indonesian isolates of *C. heterostrophus*, DNA sequencing of partial small subunit region of rDNA of SM-1 presumably representing the Indonesian isolates was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) with the ABI PRISM 37 (Applied Biosystems, USA) sequencer according to the manufacturer's recommendation. Sequencing primers used included NS3 and NS4 (Table 2) derived from conserved regions of the 18S rRNA genes of some fungal species (Fig. 1). Phylogenetic tree of the isolate was constructed using Treecon Software as suggested by the manufacturer.

Results and Discussion

The primer pair of NS3 and NS6 was able to produce amplification fragment in all of the isolates tested. A single product of estimated 907 bp was observed in the PCR product pattern (Fig. 2). As shown in the figure, the primers exhibited excellent specificity for amplification of the target genes by directing a product of the anticipated size. However, as expected, the PCR amplification was unable to exhibit genetic variations within the *C. heterostrophus* species. Restriction enzymes *Hha*I and *Rsa*I employed following the PCR process did not seem to cut the PCR product. No digested products of the two restriction endonucleases could be observed. Digestion with *Hae*III, on the other hand, produced two detectable fragments of 500 bp and 150 bp, respectively, but also failed to detect polymorphisms (data not shown).

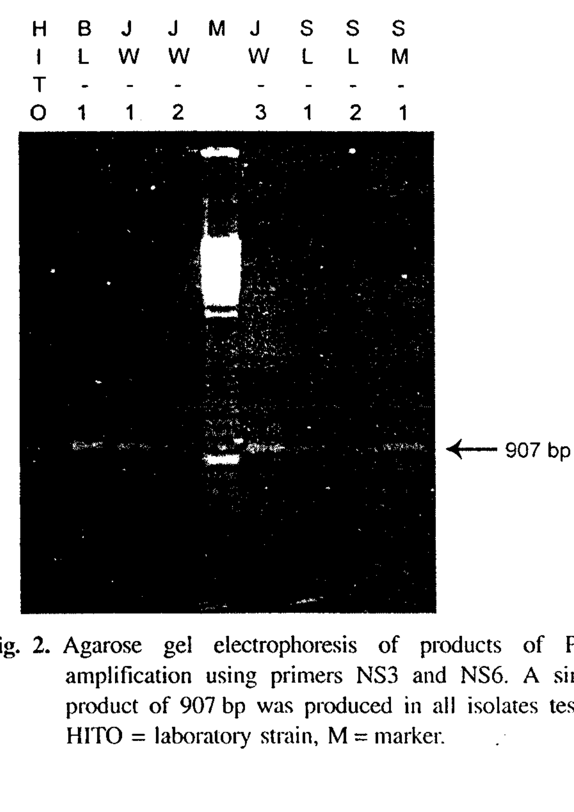

Fig. 2. Agarose gel electrophoresis of products of PCR amplification using primers NS3 and NS6. A single product of 907 bp was produced in all isolates tested. HITO = laboratory strain, M = marker.

The present PCR-RFLP analysis did not detect genetic variations in the Indonesian isolates of *C. heterostrophus*. Although the relatively small size of the PCR product and restriction enzymes employed may have also contributed to the result, the absence of polymorphisms could have been due to the fact the fungal isolates tested constitute similar rDNA genes. In a PCR amplification attempt, Jones and Dunkle (1993) were able to separate races of *Cochliobolus carbonum*, a closely related species of *C. heterostrophus*, using arbitrary primers. The primer pair of ITS-2 and ITS-3 (White *et al.*, 1990) has been combined with ITS-1F and ITS-4B primers designed by Gardes and Bruns (1993) to differentiate *Melampsora* species (Nakamura *et al.*, 1998). Similarly, Chee and Kim (2002) recognized three different varieties within the ascomycetous black yeast *Exophiala jeanselmei* based on the PCR-RFLP techniques. In deed, the PCR-RFLP has for some time been developed and used as new tools for detection, identification, and phylogenetic studies of different fungal species (Landvik *et al.*, 1998; Nakamura *et al.*, 1998).

Following failure of the PCR-RFLP analyses to detect genetic variations within the isolates of *C. heterostrophus* collected, DNA sequencing of partial small subunit region of the 18S rRNA was performed on two of the isolates to confirm the results of the PCR-RFLP analyses. Consis-

CGCAACGGNTTGAANCCTNNGNTNAACNGCGTATATTAAAGTTGTTGCAGTTAAAAAGCTCGTAG
TTGAAACTTGNNNCTGGCTGGCGGANCCGCCTCACCGCGTGCACTCGTCCGGCCGGNCCTTCCTTC
TGAAGAACCTCATGCCCTTTACTGGGCGTGTTGGGGAATCAGGACTTTTACTTTGAAAAAATTANA
GTGTTCAAAGCAGGCCTTTGCTCGAATACGTTAGCATGGAATAATAAAATAGGGCCGNGCGNTTC
TATTTTGTTGGTTTCTAGAGACGCCGCAATGATTAACAGGAACAGTCGGGGGCATCAGTATTCAGT
TGTCAGAGGTGAAATTCTTGGATTTACTGAAGACTAACNACTGCNAAAGCATTTGCCAAGGANGT
TTTCATTAATCACCGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGCCGCAGCCNTAACC
GTAAACTATGCCGACTAGGGATCGGCCCGANGCTCNTTTTCTGACTCGCTCGNCNCCTTANGAGA
AAATTAANGTTTTTGNGTTCTGNCGCCANNATGNCCCCNNGGCC

Fig. 3. Nucleotide sequence of partial small subunit of the 18S rRNA gene of the Indonesian *Cochliobolus heterostrophus* isolated in this study represented by isolate SM-1. The sequence is deposited at the GenBank under accession number AY147270.

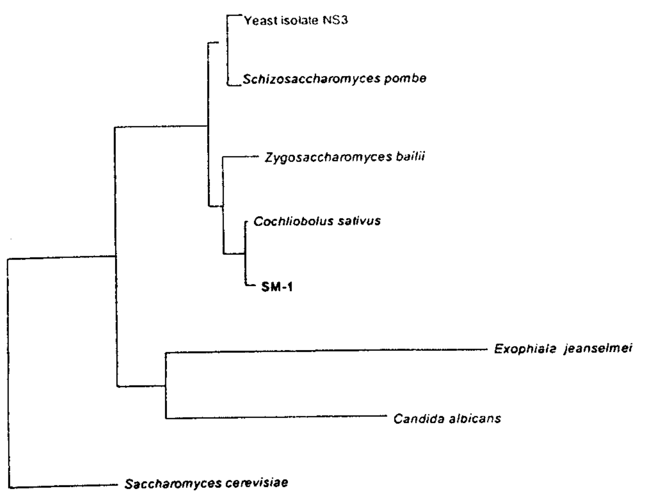

Fig. 4. Phylogenetic position of the Indonesian isolates of *Cochliobolus heterostrophus* collected in the present research represented by isolate SM-1.

tent with the results of the PCR-RFLP analyses, no appreciable variations of nucleotide sequences of the region were observed. Thus, it could be assumed that the isolates currently tested constitute similar rDNA genes. Based on the sequence of isolate SM-1 (Fig. 3), a phylogenetic tree (Fig. 4) was constructed. The figure revealed that the Indonesian *C. heterostrophus* represented by the isolate was phylogenetically located near *Cochliobolus sativus* belonging to the same genus.

Despite the fact that genetic variations within the Indonesian *C. heterostrophus* tested could not be confirmed in this study, the present results should provide a strong basis for investigations on other biological aspects of host - pathogen interactions in the pathosystem which are certainly required for possible development of transgenic corn plants resistant to the pathogens. It is also very clear that more works have yet to be attempted for better understandings of this corn ñ southern leaf blight pathosystem.

Acknowledgments

This work was funded by the DCRG-URGE Project (Contract No. 008/DCRG/URGE/2000), DGHE, Ministry of National Education of the Republic of Indonesia, Jakarta, Indonesia. The authors wish to thank E. Puspitasari and R. Rina for excellent technical assistance.

References

Alcorn, J. L. 1983. Generic concepts in *Drechslera*, *Bipolaris* and *Exserohilum*. *Mycotaxon* **17**: 1-86.

Bronson, C. R., Taga, M. and Yoder, O. C. 1990. Genetic control and distorted segregation of the T-toxin production in field isolates of *Cochliobolus heterostrophus*. *Phytopathology* **80**: 819-823.

Bruns, T. D., White, J. and Taylor, J. W. 1991. Fungal molecular systematics. *Ann. Rev. Ecol. Syst.* **22**: 525-564.

Chee, H. Y. and Kim, Y. K. 2002. Molecular analysis of *Exophiala* species using molecular markers. *Mycobiology* **30**: 1-4.

Gafur, A., Tanaka, C., Shimizu, K., Ouchi, S. and Tsuda, M. 1998. Molecular analysis and characterization of the *Cochliobolus heterostrophus* β-tubulin gene and its possible role in conferring resistance to benomyl. *J. Gen. Appl. Microbiol.* **44**: 217-223.

_____, _____, Ouchi, S. and Tsuda, M. 1997. A PCR-based method for mating type determination in *Cochliobolus heterostrophus*. *Mycoscience* **38**: 455-458.

Gardes, M. and Bruns, T. D. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Mol. Ecol.* **2**: 113-118.

Jones, M. J. and Dunkle, L. D. 1993. Analysis of *Cochliobolus carbonum* races by PCR with arbitratory and gene-specific primers. *Phytopathology* **83**: 366-370.

Landvik, S., Kristiansen, R. and Schumacher, T. 1998. Phylogenetic and structural studies in the Thelebolaceae (Ascomycota). *Mycoscience* **39**: 49-56.

Nakada, M., Tanaka, C., Tsunewaki, K. and Tsuda, M. 1994. RFLP analysis for species separation in the genera *Bipolaris* and *Curvularia*. *Mycoscience* **35**: 271-278.

Nakamura, H., Kaneko, S., Yamaoka, Y. and Kakishima, M. 1998. Differentiation of *Melampsora* rust species on willows in Japan using PCR-RFLP analysis of ITS regions of ribosomal DNA. *Mycoscience* **39**: 105-113.

Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. Molecular Cloning - A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor.

Sanger, F., Nicklen, S. and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **74**: 5463-5467.

Sivanesan, A. 1987. Graminicolous species of *Bipolaris*, *Curvularia*, *Exserohilum* and their teleomorphs. *CAB Mycol. Pap.* **158**: 1-261.

Tae, M. S., Eom, A. H. and Lee, S. S. 2002. Sequence analyses of PCR amplified partial SSU of ribosomal DNA for identifying arbuscular mycorrhizal fungi in plant roots. *Mycobiology* **30**: 13-17.

Tanaka, C., Kubo, Y. and Tsuda, M. 1991. Genetic analysis and characterization of *Cochliobolus heterostrophus* color mutants. *Mycol. Res.* **92**: 49-56.

_____, Nakada, M. and Tsuda, M. 1992. Electrophoretic separation of chromosomes of some graminicolous fungi. *Trans. Mycol. Soc. Jpn.* **33**: 95-102.

White, T. J., Bruns, T., Lee, S. and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315-322. *In*: PCR Protocols. Innis, M. A., Gelfrand, D. H., Sninsky, J. J. and White, J. (Eds.). Academic Press. San Diego.

Yoder, O. C. 1988. *Cochliobolus heterostrophus*, cause of southern corn leaf blight. Pp. 93-112. *In*: Genetics of plant pathogenic fungi (Advances in Plant Pathology 6). Ingram, D. D. and Williams, P. H. (Eds.). Academic Press. London.