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Short Communication

A PCR-based method for mating type determination in *Cochliobolus heterostrophus*

Abdul Gafur[1], Chihiro Tanaka[2], Seiji Ouchi[1] and Mitsuya Tsuda[2]

[1] Laboratory of Plant Pathology, Faculty of Agriculture, Kinki University, 3327–204, Naka-machi, Nara 631, Japan
[2] Laboratory of Environmental Mycoscience, Faculty of Agriculture, Kyoto University, Kitashirakawa-Oiwake-cho, Sakyo-ku, Kyoto 606–01, Japan

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A rapid and accurate method based on multiplex PCR using three different primers was developed for determining mating type in *Cochliobolus heterostrophus*. The primers of MAT113 and MAT123 were uniquely derived from *MAT1-1* and *MAT1-2*, respectively, and the primer MATcon5 is conserved in the flanking regions of the idiomorphs. The amplification product was estimated to be 702 bp (*MAT1-1*) or 547 bp (*MAT1-2*). Crossing experiments confirmed the accuracy of this method, which requires less time than the conventional determination of mating type.

Key Words—*Cochliobolus heterostrophus*; mating type; polymerase chain reaction (PCR).

One challenging problem in the genetic study of *Cochliobolus heterostrophus* (Drechs.) Drechs. (anamorph: *Bipolaris maydis* (Nisik.) Shoemaker) has been to shorten the time required for determination of mating type. Conventionally, mating type is determined by crossing strains of interest with tester strains of known mating type, although this method is time-consuming (it takes approximately 3 wk for pseudothecia to mature). Development of a more rapid and accurate method, presumably based on molecular approaches, is therefore useful for genetic experiments. The purpose of the study reported here was to establish and evaluate a method for determining mating type in *C. heterostrophus* based on polymerase chain reaction (PCR) amplification. Such a method should be accurate and require shorter time than the conventional determination of the mating type of *C. heterostrophus*.

In *C. heterostrophus*, mating type is controlled by two alternate forms of alleles called idiomorphs, *MAT1-1* and *MAT1-2* (Yoder, 1988). The idiomorphs, consisting of non-homologous sequences that each encode a single *MAT* specific transcript, have been characterized (Leubner-Metzger et al., 1992; Turgeon et al., 1993, Wirsel et al., 1996). Based on the nucleotide sequences of the *MAT* idiomorphs and their flanking regions (Turgeon et al., 1993), we designed oligonucleotide primers that would amplify specific region within each gene. DNA sequences of the *MAT1-1* and *MAT1-2* were aligned by CLUSTAL W (Thompson et al., 1994). While the sequences of the primers MAT113 and MAT123 were uniquely derived from the idiomorphs of *MAT1-1* and *MAT1-2*, respectively, that of the primer MATcon5 is conserved in the flanking regions of the idiomorphs (Table 1). The primers were either used simultaneously in a multiplex PCR or in pairs. The primer pair of MATcon5 and MAT113 was expected to amplify a product of 702 base pairs (bp) in the *MAT1-1*; the primer pair of MATcon5 and MAT123 a product of 547 bp in the *MAT1-2*.

The strains used in this study are listed in Table 2. HITO7711 and MASHIKI2-2 (Tanaka et al., 1991) are wild type stock cultures. The strains were inoculated in 3 ml of diluted V-8 medium broth (Nakada et al., 1994) and incubated at 25°C on a shaker for 1 to 2 d. The cultured mycelia were recovered by centrifugation and lyophilyzed. DNA was extracted by the mini-preparation method of Nakada et al. (1994).

DNA amplifications by PCR were performed in standard 50-μl reactions containing 10 pmol of each primer, 2 μl of 2.5 mM deoxynucleotide triphosphates (dNTPs), 5 μl of 10 X PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$), 1 unit of *Taq* DNA polymerase (Takara Shuzo) and approximately 200 ng of genomic DNA as a template. All reagents were preassembled in a bulk mix from which a 49-μl aliquot was dispensed into each of the 500-μl microcentrifuge tubes before template DNA was added in 1-μl volumes. Negative control reactions, in which distilled water was added instead of template DNA, were also run in all experiments. The mixtures were reacted on a TaKaRa Thermal Cycler with the following thermal profile: initial denaturation at 94°C for 60 s followed by 25 repeated cycles of melting, annealing and extension of DNA at 94°C for 30 s, 63°C for 60 s and 72°C for 90 s, respectively. In the last cycle, the extension step was increased to 7 min to complete primer elongation. The PCR products were then analyzed by the agarose gel electrophoresis in 1 X Tris-

Table 1. Primer pairs used for mating type determination in *Cochliobolus heterostrophus*.

Primer pair[a]	Tm (°C)	Expected product (bp)	Idiomorph of amplified fragment
MATcon5/ MAT113	62.4/ 62.6	702	*MAT1-1*
MATcon5/ MAT123	62.4/ 63.4	547	*MAT1-2*

a) The sequences of primers MATcon5, MAT113 and MAT123 are 5′-TCTTTGTTTTCCTGTGACTGCCTGTTG-3′, 5′-AGGTAGTTTGAGGTGAGGGCAGATGATG-3′ and 5′-CTGGGCTGATTGGGGGCTTGATAC-3′, respectively. The primers were derived from the DNA sequences of *MAT1-1* and *MAT1-2* idiomorphs of *C. heterostrophus* and their flanking regions provided by Turgeon et al. (1993) (GenBank accession numbers X68399 and X68398, respectively).

Table 2. Strains of *Cochliobolus heterostrophus* used in this study and their PCR products and mating type.

Strain No.	Origin	Amplified fragment[a]			Mating type[b]
		MATcon5/ MAT113/ MAT123	MATcon5/ MAT113	MATcon5/ MAT123	
HITO7711	Laboratory stock culture	547 bp	—	547 bp	*MAT1-2*
MASHIKI2-2	Laboratory stock culture	702 bp	702 bp	—	*MAT1-1*
PRE051	Laboratory stock culture	547 bp	—	547 bp	*MAT1-2*
PRE058	Laboratory stock culture	547 bp	—	547 bp	*MAT1-2*
PRE059	Laboratory stock culture	547 bp	—	547 bp	*MAT1-2*
PRE068	Laboratory stock culture	547 bp	—	547 bp	*MAT1-2*
PRE051-104	Progeny of PRE051 × MASHIKI2-2	702 bp	702 bp	—	*MAT1-1*
PRE058-302	Progeny of PRE051 × MASHIKI2-2	702 bp	702 bp	—	*MAT1-1*
PRE059-020	Progeny of PRE051 × MASHIKI2-2	702 bp	702 bp	—	*MAT1-1*
PRE068-201	Progeny of PRE051 × MASHIKI2-2	702 bp	702 bp	—	*MAT1-1*
PRE068-101	Progeny of PRE051 × MASHIKI2-2[c]	702 bp	702 bp	—	*MAT1-1*
PRE068-102	Progeny of PRE051 × MASHIKI2-2[c]	702 bp	702 bp	—	*MAT1-1*
PRE068-103	Progeny of PRE051 × MASHIKI2-2[c]	702 bp	702 bp	—	*MAT1-1*
PRE068-104	Progeny of PRE051 × MASHIKI2-2[c]	547 bp	—	547 bp	*MAT1-2*
PRE068-105	Progeny of PRE051 × MASHIKI2-2[c]	547 bp	—	547 bp	*MAT1-2*
PRE068-106	Progeny of PRE051 × MASHIKI2-2[c]	702 bp	702 bp	—	*MAT1-1*
PRE068-107	Progeny of PRE051 × MASHIKI2-2[c]	547 bp	—	547 bp	*MAT1-2*
PRE068-108	Progeny of PRE051 × MASHIKI2-2[c]	547 bp	—	547 bp	*MAT1-2*

a) Amplification products of either estimated 702 base pair (bp) or 547 bp were obseved when all three primers or the correct primer pairs were used. —: no product was obtained.

b) Mating types except those for HITO7711 and MASHIKI2-2 (Tanaka et al., 1991) were determined by the method presently described.

c) Sister strains originated from a single ascus.

acetate-EDTA (TAE) buffer (40 mM Tris-acetate (pH 8.0) and 1 mM EDTA) (Sambrook et al., 1989).

When all three oligonucleotide primers, MATcon5, MAT113 and MAT123, were added simultaneously to a multiplex PCR mixture, two distinctive patterns of amplification products were obtained from PCR amplification of the *C. heterostrophus* DNA tested (Fig. 1). This enabled us to determine the mating type of all strains tested. As expected, a single product of estimated 702 bp, amplified supposedly by the primer pair of MATcon5 and MAT113, was observed in one of the two patterns. This corresponds to the *MAT1-1* idiomorph, from which the MAT113 primer was derived. In the other pattern, a unique product of predicted 547 bp, presumably amplified by the primer pair of MATcon5 and MAT123, was obtained. The 547-bp product was attributed to the *MAT1-2* idiomorph, the origin of the MAT123 primer. When genomic DNAs extracted from ascospore isolates originating from a single ascus were used as templates, 4 of the 8 progenies produced the

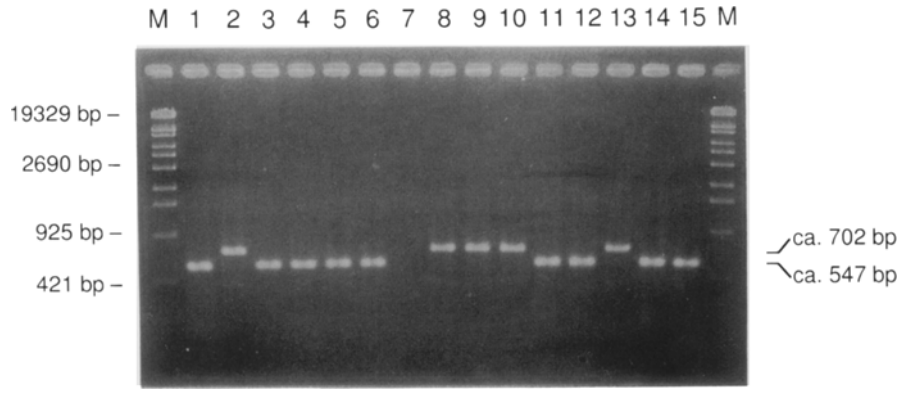

Fig. 1. Agarose gel electrophoresis of PCR amplification products using primers MATcon5, MAT113 and MAT123.
Estimated 702 base pair (bp) or 547 bp fragment was amplified when DNAs from some of the strains listed in Table 1 were used as templates. M, molecular marker (λ-DNA digested with *Sty*I); lane 1, HITO7711; lane 2, MASHIKI2-2; lane 3, PRE051; lane 4, PRE058; lane 5, PRE059; lane 6, PRE068; lane 7, negative control; lane 8, PRE068-101; lane 9, PRE068-102; lane 10, PRE068-103; lane 11, PRE068-104; lane 12, PRE068-105; lane 13, PRE068-106; lane 14, PRE068-107; lane 15, PRE068-108. Sizes of the PCR products are indicated on the right side.

predicted 702-bp fragment from the *MAT1-1* idiomorph, while the other 4 produced the anticipated 547-bp product from the *MAT1-2* idiomorph (Fig. 1, lanes 8–15).

The primer pairs of MATcon5 and MAT113 and of MATcon5 and MAT123 were then employed for production of the estimated 702-bp and 547-bp fragments from *MAT1-1* and *MAT1-2* idiomorphs, respectively. As shown in Table 2 and Fig. 2, these primer pairs exhibited complete specificity for determination of the mating types of *C. heterostrophus* by directing amplification products of anticipated 702 bp (*MAT1-1*) and 547 bp (*MAT1-2*), respectively. To further verify the specificity of the primers, template DNA was amplified using the incorrect pairs of primers. The pair of MATcon5 and MAT113 was used to amplify the *MAT1-2* idiomorph; MATcon5 and MAT123 to amplify *MAT1-1*. None of the tested DNAs led to the production of a discrete band on ethidium bromide-stained agarose gel upon amplification with the incorrect pair of primers (Table 2, Fig. 2). In all experiments, no amplification product was detected in the control reaction mixture lacking template DNA (Figs. 1, 2).

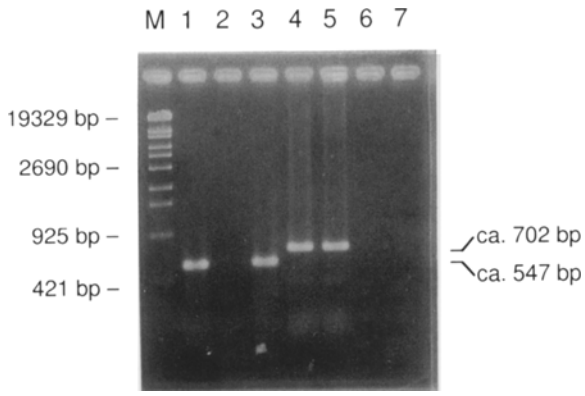

Fig. 2. Amplification products from PCR directed by correct or incorrect primer pair.
Primers used were MATcon5, MAT113 and MAT123 (lanes 1, 4 and 7), MATcon5 and MAT113 (lanes 2 and 5) and MATcon5 and MAT123 (lanes 3 and 6). M, molecular marker (λ-DNA digested with *Sty*I); lanes 1–3, HITO7711; lanes 4–6, MASHIKI2-2; lane 7, negative control. Sizes of the PCR products are indicated on the right side.

Crosses between strains whose mating types had been determined by this method was performed to verify the interpretation of the PCR amplification results. Crossing was carried out according to the method described by Ueyama and Tsuda (1975). Mature pseudothecia were observed after 3 wk of incubation. As shown in Table 3, all of the crosses between strains of different mating types developed pseudothecia. Also presented in Table 3 are the findings that no pseudothecia were produced from crosses involving strains of the same mating type. These results confirmed the accuracy of the method described here.

The utility of PCR as a specific and sensitive assay for plant pathogen identification is well documented (Henson and French, 1993). Our report on a PCR technique for determination of mating type in *C. heterostrophus* has offered an alternative to the conventional determination of mating type. The PCR-based technique provides a rapid and accurate assay to determine mating type in *C. heterostrophus*. With the PCR method described here, it is possible to determine mating type of *C. heterostrophus* in less than 3 d.

The present paper is the first report on the application of multiplex PCR technique to the determination of mating type in fungi. In view of the similarity of the overall organization of the *MAT* genes in some filamentous Ascomycetes (Turgeon et al., 1996), it is expected that this quick and accurate PCR assay will also be applicable to mating type determination in other heterothallic Ascomycete species.

Table 3. Crosses between *Cochliobolus heterostrophus* strains whose mating type had been determined by PCR-based method.

Cross	Combination of mating type	Pseudothecia formation[a)]
MASHIKI2-2 × HITO7711	*MAT1-1* × *MAT1-2*	+
MASHIKI2-2 × PRE068-101	*MAT1-1* × *MAT1-1*	−
HITO7711 × PRE068-104	*MAT1-2* × *MAT1-2*	−
PRE068-102 × PRE068	*MAT1-1* × *MAT1-2*	+
PRE068-103 × PRE058-302	*MAT1-1* × *MAT1-1*	−
PRE068-105 × PRE051	*MAT1-2* × *MAT1-2*	−
PRE068-106 × PRE068-107	*MAT1-1* × *MAT1-2*	+
PRE068-106 × PRE051-104	*MAT1-1* × *MAT1-1*	−
PRE058 × PRE068-108	*MAT1-2* × *MAT1-2*	−
PRE068-106 × PRE068-108	*MAT1-1* × *MAT1-2*	+
PRE068-106 × PRE059-020	*MAT1-1* × *MAT1-1*	−
PRE059 × PRE068-108	*MAT1-2* × *MAT1-2*	−

a) +: pseudothecia were observed; −: pseudothecia were not observed.

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