Cytological Karyotyping of Three *Cochliobolus* spp. by the Germ Tube Burst Method

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ABSTRACT

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Cytological karyotypes with mitotic metaphase chromosomes were analyzed for *Cochliobolus heterostrophus*, *C. carbonum*, and *C. sativus* by the germ tube burst method (GTBM). Prior to karyotyping, procedures of GTBM suitable to *Cochliobolus* were established by examining several crucial conditions such as incubation period of conidia. The estimated chromosome numbers of *C. heterostrophus* and *C. carbonum* were $n = 15$ or 16 and $n = 13$ or 15 depending on the strains, respectively. In *C. sativus*, $n = 15$ was estimated. Morphological information of chromosomes including chromosome size and a threadlike-specific structure

representing the nucleolar organizing region was also obtained. Our results for some standard strains are in agreement with previous estimates by pulsed field gel electrophoresis (PFGE) or PFGE coupled with restriction fragment length polymorphism genetic linkage analysis, but inconsistent with the previous estimates for other strains by conventional light microscopic cytology. Additionally, PFGE analysis of *C. heterostrophus* strains indicated that chromosome number was not determinable solely by PFGE, which is hampered by comigration and clumping of DNA bands.

Additional keywords: genome, Northern corn leaf blight, Southern corn leaf blight.

The genus *Cochliobolus* comprises major fungal pathogens of gramineous crops and weeds. Among species of the genus, the Southern corn leaf blight pathogen *C. heterostrophus* (Drechs.) Drechs. (anamorph, *Bipolaris maydis* [Nisikado & Miyake] Shoem.) and the Northern corn leaf spot pathogen *C. carbonum* Nelson (anamorph, *Bipolaris zeicola* [Stout] Shoem.) have been intensively researched because conventional genetic analysis through sexual crossing is feasible and because they produce host-specific toxins (13,24,25). Investigations of the two species have significantly contributed to our understanding of fungal pathogenesis and of mating systems in filamentous ascomycetes (21,25).

Karyotype information is essential to understanding the genetic nature of the organism concerned. In *C. heterostrophus*, primary karyotype data obtained by conventional light microscopy on the meiosis provided an estimated chromosome number, $n = 8$ (5). This estimation was initially supported by an analysis using pulsed field gel electrophoresis (PFGE) (20), but a later study by PFGE combined with restriction fragment length polymorphism (RFLP)-based genetic linkage analysis led to chromosome number estimates of $n = 15$ or 16 depending on the strains (9,22). In *C. carbonum*, $n = 13$ was proposed for a certain race 1 strain based on PFGE analysis (1), whereas cytological observations that support this estimate have not been reported so far. Thus, our knowledge of the karyotypes of these two species is inconclusive. This situation is in marked contrast to those of model fungi such as *Neurospora crassa* and *Saccharomyces cerevisiae*, in which comprehensive karyotypes have been established by different methodologies including PFGE, conventional light microscopy, electron microscopy, and genetic linkage analysis (26). To reach a solid conclusion regarding karyotypes, further analyses by different methods followed by comparison and integration of the data are needed for the *Cochliobolus* spp.

The objective of this study was to determine the cytological karyotypes of *C. heterostrophus*, *C. carbonum*, and *C. sativus* (Ito and Kurib.) Drechs. ex Dastur (anamorph, *Bipolaris sorokiniana* [Sacc. in Sorokin] Shoem.). Instead of conventional meiotic cytology, we used a rather new method called the germ tube burst method (GTBM) (15,17). GTBM utilizes the burst of conidial germ tubes to discharge mitotic metaphase chromosomes outside germling cells and spread them on the slide. This method has been applied to several plant pathogenic fungi (2,15–18) and is superior to the conventional cytology for chromosome counting as well as in characterizing the morphology of chromosomes (18).

In this study, we examined conditions of GTBM specific to *Cochliobolus* spp., and thereby established the procedures fit for species of this genus. With this GTBM, cytological karyotypes of the three species were successfully determined. In addition, electrophoretic karyotypes of *C. heterostrophus* were analyzed by PFGE. The results obtained in this study, we believe, represent the most reliable karyotypes from cytology for the genus *Cochliobolus*.

MATERIALS AND METHODS

Strains. The fungal strains used in this study are listed in Table 1. For the four strains, Hm540, B30.A3.R.45, HITO7711, and SB111, karyotypes from PFGE have been already reported (1,20,22). All strains, except B30.A3.R.45, were routinely cultured on complete medium (CM) (11). CMX medium (22) was used for B30.A3.R.45 in place of CM. Massive conidiation was achieved for each strain by culturing under continuous near-ultraviolet light for 5 days at 24°C.

Time course analysis of metaphase frequency. Conidia of *C. heterostrophus* strain Hm540 were harvested in water containing Tween 20 (0.02%, wt/vol), washed twice with potato dextrose broth (PDB) (Difco Laboratories, Detroit) by centrifugation, and suspended in PDB at a concentration of 4×10^4 per ml. A drop of conidial suspension (100 μ l) was placed on a slide and incubated in high humidity at 25°C. The slides were successively sampled at 10-min intervals, fixed with 3% formaldehyde in sodium phos-

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phate buffer (0.1 M, pH 7.0) for 3 min, washed with the buffer, and air-dried at room temperature for at least 30 min. The specimens prepared were stained for cell walls with calcofluor (20 µg/ml in distilled water) for 5 min, then nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (1 µg/ml) in antifade mounting solution (8). Observation was made with an epifluorescence microscope (BHS-RFC with a BH2-DMU cube; Olympus, Tokyo) for evaluating both the germination rate of conidia and the frequency of metaphase nuclei in the population of germ tube cells, hereafter called metaphase frequency (MF). MF was measured by the following formula; percent of MF = number of apical cells with metaphase nuclei × 100 / total number of apical cells observed. Only apical cells were subjected to the examination because they are the main part of germ tubes to be burst and serve as the source of cells for discharged nuclei in GTBM. The individual cell, not individual nucleus, was used as a counting unit, because mitotic stages of nuclei in the same cell were nearly synchronized. Measurements of MF were repeated three times with at least 350 apical cells each, and the combined results were used to calculate the final value of MF. The germination rate of conidia was also measured three times with at least 200 conidia each, and the results were combined.

Hydroxyurea and thiabendazole treatment. Conidia of strain Hm540 were harvested in water containing 0.02% Tween 20, washed twice with PDB by centrifugation, and suspended at 1.5×10^5 conidia per ml in PDB. For hydroxyurea (HU) treatment, 100 µl of the conidial suspension amended with 50 mM HU was dropped on a slide and incubated under high humidity for 150 min at 25°C. The slides were transferred into water in a staining jar and shaken at 90 rpm for 5 min to wash away the HU. After removing excess water, 200 µl of PDB was added to each specimen and incubation was resumed. The slides were sampled for fixation at 10-min intervals from the resumption of incubation. For thiabendazole (TBZ) treatment, a 100-µl conidial suspension was placed on a slide and incubated for 100 min at 25°C, followed by washing with water to remove PDB. Then, the incubation medium was replaced with 200 µl of PDB containing 10 or 50 µg/ml of TBZ, and further incubated for 60 min at 25°C. In both treatments, fixation and staining of samples were done, as in the time course experiment, without chemical treatment. The measurement of MF was made twice with at least 250 apical cells each, and the results were combined and calculated as described previously.

Chromosome preparation. GTBM was used to prepare chromosome specimens, with some modifications of original procedure (15,17). The conditions specific to *Cochliobolus* were as follow. A 100-µl sample of conidial suspension (1.5×10^5 conidia per ml), prepared as in the time course analysis of MF, was placed on a slide and incubated under humid condition for 2 to 3.5 h at 25 to 27°C, depending on the species. The slides were then dipped in water to remove PDB, immersed in fixative (99% methanol/glacial acetic acid = 22:3, vol/vol) for 20 min at room temperature, and flame dried. The fixed specimens were stained with DAPI and observed under an epifluorescence microscope.

Fluorescence microscopy and photography. Fluorescence observations were made with an epifluorescence microscope, and photographs were taken on 400 ASA/ISO color print film (Fujicolor Super HG400; Fuji Film Inc., Tokyo). The color images on the printed pictures were transformed to black and white images by the personal computer software, Photoshop (version 5.0; Adobe Systems Inc., San Jose, CA).

Preparation of chromosomal DNA. Chromosomal DNAs (ChDNAs) for PFGE were prepared in *C. heterostrophus* strains Hm540 and B30.A3.R.45A. Conidia from 1-week-old culture were harvested in sterile distilled water containing Tween 20 (0.05%) and filtered through one layer of Kimwipe (Kimberly-Clark Corp., Dallas) to remove hyphal debris. The conidial suspension was added to 100 ml of liquid CM in a 300-ml flask (final concentration, 1×10^6 conidia per ml) and incubated on a reciprocal shaker (110 strokes per min) for 12 to 15 h at 27°C. The germings were vacuum filtrated on a Whatman No. 4 filter paper, washed with 0.7 M NaCl, suspended in 6 to 10 ml of filter-sterilized enzyme solution containing 2 mg of Novozym 234 (Novo Biolabs, Bagsvaerd, Denmark), 3 mg of Kitalase (Wako Pure Chemical Inc., Osaka, Japan), and 3 mg of β-glucuronidase (type HA-4; Sigma-Aldrich, St. Louis) per milliliter of 0.7 M NaCl, and incubated on a shaker (65 to 75 strokes per min) for 2 to 4 h at 30°C for protoplasting. Protoplasts were filtered through four layers of Kimwipe and collected by centrifugation at $550 \times g$ for 10 min, and the pellet was washed twice with 0.7 M NaCl by centrifugation ($550 \times g$ for 10 min). The final protoplast pellet was suspended in SE (1 M sorbitol, 50 mM EDTA, pH 8) at $\approx 2 \times 10^8$ cells per ml, embedded in 0.5% low melting agarose (Bio-Rad Laboratories, Hercules, CA) in SE, and incubated in a (0.5 M EDTA, 10 mM Tris-HCl at pH 8, and 1%, wt/vol, sodium lauroyl sarcosinate) to lyse for 14 h at 37°C. After rinsing three times in 50 mM EDTA (pH 8) for 30 min each at 37°C, samples were stored in this solution at 4°C.

Pulsed field gel electrophoresis. ChDNAs were separated in 0.9% agarose gels (PFGE certified, CHEF-DR II; Bio-Rad) at 12°C in 0.5× Tris-borate-EDTA buffer (14). Voltage, switching intervals, and total running times were: 1.5 V/cm with a ramped 3,600- to 1,800-s switching interval for 72 h, 1,800- to 1,300-s interval for 36 h at the same voltage, 1.8 V/cm with a 1,300- to 800-s interval for 42 h, and 2.4 V/cm with a 800- to 600-s interval for 36 h. After finishing electrophoresis, gels were stained with ethidium bromide (0.5 µg/ml) for 1 h, destained in water for 1 h, and photographed with UV illumination.

RESULTS

GTBM for *Cochliobolus*. In GTBM, nuclei were discharged outside germ tube cells to spread on the surface of slide. A high frequency of metaphase nuclei in the spreads is desirable to perform karyotyping by GTBM. Because our experience with other fungi indicated that the incubation period of conidia is important to attain such condition, a time course of the germination rate of conidia and MF in germ tube cells was analyzed (Fig. 1).

TABLE 1. Isolates and strains of three *Cochliobolus* spp. used in this study and their estimated chromosome numbers

Species	Isolate or strain	Race	Locality	Source	Chromosome number
<i>C. heterostrophus</i>	Hm540	O	North Carolina, U.S.	C. R. Bronson	15
	B30.A3.R.45	T	Laboratory strain	C. R. Bronson	16
	C2	T	Laboratory strain	O. C. Yoder	16
	C4	T	Laboratory strain	O. C. Yoder	16
	HITO7711	O	Kumamoto, Japan	M. Tsuda	15
<i>C. carbonum</i>	SB111	1	Iowa, U.S.	J. D. Walton	13
	SB114	2	Iowa, U.S.	J. D. Walton	13
	BZ1207	3	Tochigi, Japan	T. Tsukiboshi	15
	BZ1801	3	Miyagi, Japan	T. Tsukiboshi	15
<i>C. sativus</i>	CS4	-	Hokkaido, Japan	J. Soma	15

Conidia started to germinate at 20 min of incubation, and the germination rate exceeded 90% at 70 min. Germ tubes became septated at 60 to 90 min, giving rise to multinucleate apical and basal cells. In the germ tubes undergoing successive septation, mitotic stages of nuclei of the same cell were nearly synchronized, and the metaphase could be distinguished from others based on the aggregation of condensed chromosomes (Fig. 2). During the 3-h incubation, the MF sharply fluctuated with the first and second peak appearing at 70 and 130 min, and the following first and second trough at 100 and 160 min, respectively (Fig. 1). These intervals indicated that fluctuation of MF was periodic with

Fig. 1. Time course of metaphase frequency (MF) and germination rate during incubation of *Cochliobolus heterostrophus* Hm540. MF was measured at 10-min intervals between 60- and 180-min incubation. The germination rate was measured at 10-min intervals up to 120-min incubation. Percent of MF = number of apical cells with metaphase nuclei $\times$ 100 / total number of apical cells.

Fig. 2. Synchronous nuclear division in the germ tube cell of *Cochliobolus heterostrophus*. A part of the germ tube of strain Hm540 is shown. All nuclei in the apical (upper) cell are at metaphase. The proximal (lower) cell contains nuclei at interphase. The specimen was double stained with calcofluor and 4',6-diamidino-2-phenylindole. Scale bar = 5 μ m.

a 60-min cycle. It was evident from this time-course analysis that incubation period of conidia, or the timing of the burst of germ tubes, is crucial to gain high MF, and the optima were 70 and 130 min for *C. heterostrophus*.

In order to raise MF, synchronization of mitosis was attempted by using the DNA synthesis inhibitor HU, to arrest the cell cycle of germ tube cells at the entry of S phase, then washing the cells to resume the cell cycle. In the first 20 min after removal of HU, the MF was very low but rose rapidly to $\approx$ 18% at 30 min after removal of HU (Fig. 3). Because the maximum MF was $\approx$ 12% without HU treatment (Fig. 1), HU treatment appeared to increase MF. TBZ, a microtubular inhibitor effective for metaphase-arrest in some microbes, was also tested, but was ineffective for *Cochliobolus* (data not shown).

Fixative, which serves for bursting germ tubes as well as fixing chromosomes in GTBM, was also examined. According to our experience, the burst rate of germ tube was greatly dependent on the kind of fixative. After testing various fixatives, we found that the mixture of methanol and acetic acid in the ratio of 22:3 was useful for all strains of the three species.

The specific conditions of GTBM established for the three species were as follows. The incubation temperature was 25°C for *C. heterostrophus* and *C. carbonum* and 27°C for *C. sativus*. Without HU treatment, the incubation period was $\approx$ 2 h for *C. heterostrophus* and $\approx$ 3 h for both *C. carbonum* and *C. sativus*. With HU treatment, incubation was continued for 30 min after removal of HU.

Chromosome number. Ten strains of the three species were examined for their chromosomes numbers by specimens both with and without HU treatment. Although various mitotic stages from prophase to telophase were found in the spreads, nuclei with condensed chromosomes probably at metaphase were selected. For chromosome counting, only nuclei with a reasonably large number (approximately 10 or more at first glance) of well spread chromosomes were used. The other nuclei with overlapping or apparently small numbers of chromosomes were excluded from the analysis.

At first, the three standard strains, Hm540 and B30.A3.R.45 of *C. heterostrophus* and SB111 of *C. carbonum*, were intensively analyzed with more than 50 nuclei to evaluate the constancy of chromosome count by GTBM. In each strain, counts were not constant but varied in a limited range (Fig. 4). A particular count (namely, 15, 16, and 13) was conspicuously frequent in each of isolates Hm540, B30.A3.R.45, and SB111, respectively. These most frequent counts were adopted as the estimate of chromosome number of each strain. The other seven strains were similarly analyzed with more than 10 nuclei for each. The results are summarized in Table 1. The observation that C2, C4, and B30.A3.R.45 of *C. heterostrophus* shared the same chromosome

Fig. 3. Time course of metaphase frequency (MF) after release from hydroxyurea block of cell cycle in *Cochliobolus heterostrophus*. MF was measured at 10-min intervals for 100 min after release from the cell cycle block.

number was consistent with their genealogy, in that they are near-isogenic laboratory-bred strains with a common progenitor (11,22). In *C. heterostrophus* and *C. carbonum*, variation of chromosome number was limited at the species level in spite of the differences in the locality and pathogenicity of the strains used.

Compared with previous reports, our estimates of strains Hm540 and B30.A3.R.45 were consistent with those from PFGE and RFLP linkage analyses for the same two strains (22). Also, our estimate for SB111 chromosome number agreed with the estimate from PFGE (1). In contrast, our estimate of $n = 15$ for HTO7711 of *C. heterostrophus* was discrepant from $n = 8$ from PFGE analysis (20). For the rest of the strains, such direct comparison was not possible because their karyotypes are reported for the first time in this study.

Morphological features of chromosomes. Morphology of the component chromosomes was examined to obtain karyotypic information other than chromosome number. Unlike chromosomes of higher eukaryotes, neither centromeric constriction nor sister chromatids were discernible in *Cochliobolus* chromosomes under our conditions. Morphological features obtained in our observation were chromosome size and projection of a chromatin thread from the end of a particular chromosome that is called the threadlike structure and harbors the rRNA gene cluster or nucleolar organizing region (NOR) (15,17).

A constant measurement of chromosome size was not obtainable due to the varying degree of chromosome contraction dependent on the nucleus analyzed. Nevertheless, comparison of relative size was possible for chromosomes in each individual nucleus. For example, relative size based on the measurement of axial length ranged 1 to 2.8, 1 to 2.5, and 1 to 2.5 for the chromosomes of Hm540, B30.A3.R.45, SB111, and CS4, shown in Figure 5, where chromosomes are roughly aligned according to their sizes. Identification of every individual chromosome by their sizes was difficult, because size differences among similar-sized chromosomes was too small to precisely distinguish them.

A threadlike structure was found on a relatively small chromosome in each species. The length of this structure varied with the specimens, correlating with the condensation of chromosomes. This structure was distinct in *C. carbonum* strains, but less distinct or more often not detectable in *C. heterostrophus* and *C. sativus*. Thus, at least in *C. carbonum*, the threadlike structure served as a useful morphological marker of the nucleolar organizing chromosome.

Electrophoretic karyotypes. PFGE was carried out for strains Hm540 and B30.A3.R.45. Our methods of protoplasting of fungal cells and preparing agarose blocks were different from precedents (22), but worked very well. Using our protoplasts-embedded agarose blocks, reasonably sharp chDNA bands were separable by PFGE with faint background smear. Among several running conditions tested, a specific condition separated chDNAs fairly well over a wide range from ≈ 4 to 1 megabases (Fig. 6A). Although different apparatus and running condition were used, the banding patterns and sizes of separated chDNAs were consistent with the results of Tzeng et al. (22) for the same two strains. Because comigration and clumping of bands hampered complete karyotyping of both strains under the running condition previously mentioned, attempts were made to resolve these bands into discrete single bands. Further resolution, however, could not be achieved with conditions that preferentially separate smaller or larger bands (Fig. 6B and C). Curiously, the clumped bands (Fig. 6A to C, closed circles) maintained smearlike appearances under different running conditions. Overall, the PFGE analysis provided only minimum estimates of chromosome numbers, that is, 13 and 14 for Hm540 and B30.A3.R.45, provided that each brighter band represents two comigrating chromosomes of the same size.

DISCUSSION

GTBM has been applied to a very limited number of plant pathogens such as *Botrytis cinerea* (15–17), *Alternaria alternata* (2,17), and *Nectria haematococca* (18,19). Because this method has never been used in *Cochliobolus*, the procedures that allow efficient karyotyping of the three *Cochliobolus* spp. were newly established in this study. The GTBM used here will be applicable to other *Cochliobolus* spp. with minor modifications. The original GTBM developed for *Botrytis cinerea* was combined with HCl-Giemsa staining of chromosomes (15,16), whereas we used DAPI staining and fluorescence microscopy to visualize chromosomes. Owing to the high-binding specificity of DAPI to DNA and needlessness of additional treatments such as acid hydrolysis of the specimens, this fluorescence staining is reliable and time saving compared with other staining techniques. Therefore, fluorescence microscopy is recommended for workers who use GTBM for karyotype studies.

Chemical treatment is often used to increase MF for karyotyping in many organisms. In this study, however, a large increase of

Fig. 4. Chromosome counts of the standard strains of *Cochliobolus heterostrophus* and *C. carbonum* by the germ tube burst method. Histograms were made from 118, 51, and 67 counts for Hm540, B30.A3.R.45, and SB111, respectively.

MF was not achieved by treatment with either HU and TBZ. Similarly, only a slight increase of MF has been accomplished in TBZ-treated *Schizosaccharomyces pombe*, whereas utilization of a cell-division cycle mutant was much more efficient than TBZ treatment (23). In *C. heterostrophus*, the maximum MF without chemical treatment was $\approx 12\%$, which was much higher than that ($\approx 6\%$) obtained in another multinucleate fungus *Botrytis cinerea* (15). Because this value was acceptably high, there is no need for additional treatment to raise MF in *C. heterostrophus*. The lack of the effect of TBZ in this study was probably because this fungus has β -tubulin gene conferring natural benzimidazole resistance (4).

The intensive chromosome counting of the three strains of *C. heterostrophus* and *C. carbonum* revealed the occurrence and extent of variation of the counts obtained by GTBM. Because such variation is probably innate in GTBM, estimation of chromosome number by this method inevitably has to be made on the frequency analysis of the count as we did. This kind of variation in chromosome counting is not unique to GTBM, but rather common in cytological karyotyping of many organisms. For instance, using a spreading method different from GTBM, Kuroiwa et al. (10) observed similar variation of chromosome counts in *Saccharomyces cerevisiae* and correctly determined the chromosome number by adopting the most frequent count. Two main factors are likely causes of variation of counts. One is the artifacts, such as dropping away of some chromosomes from slides, and the other is the

asynchronous separation of sister chromatids. In addition, a part of the variation could be a manifestation of somatic aneuploidy due to aberrant chromosome behavior such as mitotic nondisjunction.

Our estimation of chromosome number for the three strains is consistent with the previous estimation for the same strains by PFGE or PFGE and RFLP genetic linkage analysis (1,22). This consistency strengthens the reliability of both estimations, and in combination, these two lines of investigation represent more comprehensive karyotypes of *Cochliobolus*. In contrast, our estimation is apparently inconsistent with those by conventional light microscopic cytology. Conventional meiotic or mitotic cytology provided $n = 8$ for *C. heterostrophus* (5) and $n = 6$ to 8 for *C. sativus* (6,7), whereas our estimates are almost double, i.e., $n = 15$ to 16 for *C. heterostrophus* and 15 for *C. sativus*. Apparently, these discrepancies are not due to the difference of strains analyzed, but due to the methods employed. In *Nectria haematococca*, Taga et al. (18) found similar discrepancy between the estimates from GTBM or PFGE and those from conventional meiotic cytology. They attributed the discrepancy to underestimation by the conventional methods. We believe this is also the case for *Cochliobolus*. Reexamination of the past cytological data, especially of meiosis, is necessary in this genus. In this study, strain-dependent variation of chromosome number was found in *C. heterostrophus* and *C. carbonum*. According to Tzeng et al. (22), the difference between Hm540 and B30.A3.R.45 of *C. heterostrophus* is attrib-

Fig. 5. Karyotypes of selected strains of three *Cochliobolus* spp. based on mitotic chromosome specimens prepared by the germ tube burst method. The upper part in each panel shows the spread of 4',6-diamidino-2-phenylindole (DAPI)-stained metaphase nucleus with condensed chromosomes; the lower part is the size-based alignment of the chromosomes. Arrows in the figures indicate the threadlike structure with the nucleolar organizing region. Each specimen was stained with DAPI. Scale bars = 2 μ m.

utable to a B chromosome contained in the latter strain. The same B chromosome should also cause a difference between Hm540 and the other race T strains C2 and C4, because these race T strains are near isogenic to B30.A3.R.45. Although the smaller chromosome number of the two race O strains Hm540 and HTO7711 suggests that basic chromosome number of *C. heterostrophus* is not more than 15, it is still unknown if these race O strains are euploids. There remains a possibility that Hm540 and HTO7711 have some additional chromosomes and, hence, are hyperploids. In *C. carbonum*, two Japanese strains are thought to have two more chromosomes than U.S. strains have. In contrast to *C. heterostrophus*, neither B chromosome nor hyperploidy has been revealed in this species, but either situation is possible for the Japanese strains. Undoubtedly, extensive surveys with more strains are necessary to conclude the extent of variation of chromosome number and to test its possible linkage to the geographic origin or to pathogenicity of strains in *Cochliobolus* spp. Our result that chromosome numbers of the three *Cochliobolus* spp. were similar to each other reflects the close phylogenetic relationship among them that is inferred from sequence analysis (3) and also indicated by the successful interspecific cross between *C. heterostrophus* and *C. carbonum* and between *C. carbonum* and *C. sativus* (12).

Although PFGE is most frequently used for karyotype studies of filamentous fungi, it is not sufficient for karyotyping *C. heterostrophus* as shown in this study. Tzeng et al. succeeded in estimating the number and size of chromosomes of the two strains of *C. heterostrophus* by combining PFGE with RFLP linkage mapping (22). Although their work was an advance in the genetics of *Cochliobolus*, such combined analysis incorporating genetic mapping is too laborious for karyotyping many species and strains. GTBM is a convenient way of karyotyping that is espe-

cially effective for the determination of chromosome numbers of strains with similar-sized chromosomes or with large chromosomes beyond resolution of PFGE and, consequently, overcomes the disadvantage of karyotyping by PFGE. The merit of using GTBM in *Cochliobolus* is also evident in the feasibility of fluorescence in situ hybridization (FISH) on the specimens made by GTBM. As shown in other fungi (17,19), molecular cytogenetic study with FISH will enable visualization of specific chromosomes and genes and help to elucidate more detailed karyotypes of this genus.

We believe that karyotypic information from cytology is indispensable to fully understand fungal genomes. Considering that such information is scarce in plant pathogenic fungi, more efforts should be made to perform cytological karyotyping in those fungi. The joint usage of GTBM with other methods, such as PFGE, will provide an ideal karyotyping method for conidiating fungi and definitely contribute to the advance of this field.

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Fig. 6. Pulsed field gel electrophoresis for the two *Cochliobolus heterostrophus* standard strains Hm540 and B30.A3.R.45. B indicates strain B30.A3.R.45; H indicates strain Hm540; and S indicates *Saccharomyces cerevisiae* as size marker. Numbers at the left indicate DNA sizes in megabases (Mb). **A**, Chromosomal DNAs separated over a wide range under the running condition: 1.5 V/cm with a 3,600- to 1,800-s switching interval for 72 h, a 1,800- to 1,300-s switching interval for 36 h, 1.8 V/cm with a 1,300- to 800-s switching interval for 42 h, and 2.4 V/cm with a 800- to 600-s switching interval for 36 h. **B**, An attempt to separate comigrating and clumping bands as shown in **A**. Running condition: 5.5 V/cm with a 120-s switching interval for 12 h and a 180-s switching interval for 12 h. **C**, Another attempt to separate comigrating and clumping bands with running condition: 1.5 V/cm with a 3,600- to 1,800-s switching interval for 24 h, a 1,800- to 1,300-s switching interval for 60 h, 1.8 V/cm with a 1,300- to 800-s switching interval for 54 h, and 2.4 V/cm with a 800- to 600-s switching interval for 72 h. Comigration and clumping of bands are indicated by arrows and closed circles, respectively. Asterisks show the smallest comigrated bands inseparable with different running conditions.

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