Molecular and cytological karyotype of *Nectria haematococca* mating population I and inheritance mode of a 410-kb supernumerary chromosome in the genome

March, 2012

By

Ahmed Mohamed Mahmoud Ahmed

Graduate School of Natural Science and Technology

(Doctor's Course)

OKAYAMA UNIVERSITY

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By

Ahmed Mohamed Mahmoud Ahmed

A dissertation submitted to

The Graduate School of Natural Science and Technology

Okayama University

As a partial fulfillment of the requirement for the degree of

Doctor of Philosophy

Approved by:

1- Associate Prof. Dr. **Masatoki Taga**

2- Prof. Dr. **Takashi Kamada**

3- Prof. Dr. **Taku Takahashi**

SUMMARY

Nectria haematococca mating population I (MPI, anamorph, *Fusarium solani* f. sp. c*ucurbitae* race 1) is the causal agent of Fusarium crown and foot rot disease of cucurbits. It occurs worldwide, causing serious damages on the cucurbits cultivations. Although genetic studies on this fungus were ahead of genetic analysis of other plant pathogenic fungi in early 1940s, such investigations ceased after 1970.

For performing genetic analysis, irrespective of whether it is conventional or molecular, and also in pursuing whole genome sequencing, information of karyotype is the underlying basis. As to the karyotype of *N. haematococca* MPI, cytological investigations were done more than 50 years ago, in which haploid chromosome number (CN) of this fungus was estimated to be n=4, Thereafter, no additional data have been added to the cytological karyotype of this fungus.

In this study, I present a reliable karyotype of this important plant pathogen, *N. haematococca* MPI using two standard strains ATCC18098 and ATCC18099, by combining molecular and cytological karyotyping methods. In addition, a mini-chromosome discovered during the course of karyotyping was analyzed for its cytological nature and meiotic inheritance mode.

Molecular karyotyping using pulsed field gel electrophoresis (PFGE) of ATCC18098 and ATCC18099 was attempted using a standard running condition of PFGE suitable to separate chromosomes below ~ 6 Mb. Results showed apparent similarity in the banding pattern of the two strains and two middle-sized chromosome bands were clearly resolved. The two bands migrated similarly between the two strains and their sizes were estimated to be ~ 3.5 and ~ 2.5 Mb. Besides these bands, an extra small band was detected in ATCC18098 with a size of ca. 410 kb. Southern hybridization of this band with telomere repeats (TTAGGG)n from *Neurospora crassa* as a probe showed the presence of telemetric repeats within this band suggesting that this band is a mini-

chromosome rather than a huge plasmid. Large chromosomes were not separated even though further attempts were made. EKs of the two strains remained unknown in this study.

Meiotic chromosomes were observed using DAPI staining as an alternative to the traditional staining methods and its usability was proven through this study. Observing the different meiotic stages was done in the asci obtained from the cross between ATCC18098 and ATCC18099. Pachytene, which has been used for karyotyping in conventional fungal cytology, was found unsuitable for karyotyping because observation of individual chromosomes was hampered by the aggregation of elongated chromosomes. It was noticed that chromosomes in this stage showed knobs or segments on the chromosome. These structures were intensely stained with DAPI, and hence supposedly AT-rich heterochromatin. Compared to pachytene, chromosomes in the subsequent stages of meiosis I were more or less discretely separated. Nevertheless, reliable karyotyping on such stages seemed difficult due to the clumping of some chromosomes. Chromosomes in meiosis II or post-meiotic mitosis were not suitable either, for the same reasons.

Mitotic chromosomes of cytological specimens were prepared either by the germ tube burst method or by the dropping method. The dropping method was shown to have some merits in obtaining chromosome specimens at a relatively high frequency but most specimens prepared by this method were in prophase, containing stretched or elongated chromosomes with which morphological features of most chromosomes was difficult to discern. GTMB, on the other hand, enabled preparation of metaphase chromosomes that were more suitable for karyotyping in terms of the characterization of morphological features of chromosomes. Thiabendazole treatment has an advantage in obtaining relatively high and fully condensed chromosomes to enable reliable chromosome counting. Also, DAPI/PI staining highlighted chromosome regions that were differentially stained by these two dyes.

In both ATCC18098 and ATCC18099, nine chromosomes were consistently counted with an extra mini-chromosome in ATCC18098 which was proven later in this study to be supernumerary. Thus, chromosome number in this fungus for both strains was CN=9, correcting the previous chromosome count by conventional meiotic cytology. Also, detailed karyotyping for each strain was obtained using chromosome length and other features such as intensely fluorescing segments (IFSs) and the protrusion of rDNA. Comparing the two karyotypes, relative ratio of chromosome length among the complements were somewhat similar between the two strains, the chromosomes were not homologous by judging from the difference in IFS. Chromosome 5 in each strain had a rDNA protrusion at its end, thus correcting the previous report that rDNA was associated with the longest chromosome.

The mini-chromosomes firstly discovered in this study in strain ATCC18098 by PFGE and southern hybridization analyses was very small (ca. 0.4–0.6 μm in length) and the whole chromosome was stained more with DAPI than with PI, suggesting that this chromosome is relatively AT-rich. In the specimens obtained by dropping method, this chromosome had a slender rod-like shape with the terminal knob on each end during early metaphase of ca. 1.0 – 1.2 μm in length and of about 0.8 μm in length at the end of that phase. Fluorescence *in situ* hybridization (FISH) results using 410-kb chromosomal DNA band of ATCC18098 showed that the probe hybridized to the mini-chromosome in the mitotic specimen of strain ATCC18098 but not to any other chromosomes. Also, The probe hybridized to a single spot in the interphase nucleus of strain ATCC 18098, proving that the 410-kb band is linked to DNA in the nucleus, not in the cytoplasm. In either case, hybridization signals were not detected in the specimens of strain ATCC18099. These results confirmed that the 410-kb band on the PFGE gel represents the mini-chromosome detected in cytological observation and that it is not the derivative of other A chromosomes of either strain. The dot-like appearance of signal in the interphase nucleus indicates that

the mini-chromosome is not decondensed, but more or less condensed even in interphase.

The mode of inheritance of the mini-chromosome in sexual crosses was studied by tetrad and random ascospore analyses with ascospores obtained from reciprocal crossings between ATCC18098 and ATCC18099. For detecting mini-chromosomes in the progenies, microconidia-agarose plugs instead of protoplast-agarose plugs were used in PFGE. Besides the presence of the mini-chromosome, MAT and perithecial color was determined in both types of ascospore analyses to check if segregation in sexual crosses was normal for the other chromosomes. The results of tetrad analysis showed that, irrespective of whether the mini-chromosome was transmitted from female or male, each of the reconstructed tetrads showed 4:4 segregation for the occurrence and absence of mini-chromosome which suggesting that this chromosome was inherited in a Mendelian manner. In random ascospore analysis, and out of 90 ascospore progenies, chi-square test supported that progeny with and without mini-chromosome segregated in 1:1 in each cross. With 40 ascospores selected randomly from each cross, linkage analysis showed that the mini-chromosome segregated independently from either MAT or perithecial color trait. Random assortment of the mini-chromosome with the other two chromosomes was also indicated from crossing data.

In conclusion, defined cytological karyotypes of the two standard strains of *N. haematococca* MPI were obtained in this study. The information presented here should be useful not only for future genetic studies including linkage mapping and analysis of karyotype polymorphism in the populations but also for a genome project on this fungus. In addition, the 410-kb mini-chromosome discovered in this study will serve as an attractive model for studying the origin and function of fungal mini-chromosome.

ACKNOWLEDGEMENTS

In the name of Allah Most Gracious Most Merciful. All praise and thanks to Allah the exalted, the Lord of the universe, May the choicest blessings and peace of Allah be upon the last Messenger and Prophets, Muhammad, his family, Companions and all those who follow his footsteps.

I am very grateful to Associate Prof. Dr. Masatoki Taga for suggesting the point, his precise instructions through the study, for many helpful discussions, for critical reading the manuscript, for his patience and kindheartedness and for his unlimited and unconditional support during the past years.

I appreciate Prof. Kamada for his support and fruitful discussions, Prof. Takahashi Taku for his supervision and Dr. Muraguchi (Akita Prefectural University) for providing the Operon RAPD primers. I also appreciate to all former and current members of Dr. Taga' lab: Nishima, Tsuchiya, Kaneko, Songlin, Arimoto, Ogura, Nishizawa and my brother Hamid for their encouragement, support, and fun time together.

Last but not least, I want to thank my dear wife and children for unconditional love and infinite patience, my family and my family-in-law for encouragement and support, the Egyptian group in Okayama for their endless support and to all the members of Okayama muslim community.

I thank the Almighty God for His grace and mercy for without Him, I would not be able to complete this work.

This study was supported by the Ministry of higher education in Egypt.

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CHAPTER I

GENERAL INTRODUCTION

Cytogenetics of filamentous fungi

Even though fungi are classified as lower eukaryotes, they have become favored model organisms for the study of eukaryotic genetics and molecular biology. The genome organization of fungi is typical of eukaryotic system containing a nucleus and membrane-bound organelles. Within the nucleus, chromosomes are composed of DNA double helices, histones, and non-histone nuclear proteins. The marked feature of fungal genome is that it exists in haploid state except in a short period prior to meiosis and also its small size comparing to those of plants and animal beside the relatively high number of genes comparing to the genome of higher eukaryotes.

Phylogenetic analysis revealed that four distinct phyla within the fungal kingdom namely, Chrytidiomycota, Zygomycota, Basidiomycota and Ascomycota which is the largest phylum (ca. 65% of all know species). Members of subphylum Pezizomycotina include all filamentous fungi (Kirk *et al.* 2008).

In filamentous fungi, cytogenetic studies have been carried out mostly on the meiotic chromosomes such as those in asci and basidium, in which using temporary smear preparations stained with hematoxylin mixture, iron-acetocarmine or acetic lactmoid were observed by conventional light microscopy. McClintock (1945) developed the fixing and staining methods of asci of *Neurospora crassa* and identified each of the seven chromosomes at pachytene which became the favorite stage for chromosomes visualization in the following researches. While many filamentous species were examined but quality is variable from those investigations on *Neurospora* and *Coprinus* (Raju 1980, Pukkila and Lu 1985, Lu 1993, Li *et al.* 1999, Perkins 2000).

Due to the small size of mitotic chromosomes and the extremely short mitotic cycle, there are few reports using conventional light microscopic techniques about chromosome visualization and cell cycle in somatic cells such as hyphae (Robinow and Caten 1969, Crackower and Bauer 1970, Crackower 1972, Duncan and Gilbraith 1973, Singh *et al.* 1984, Borbye *et al.* 1992, Poma *et al.* 1998). Shirane *et al.* (1989) introduced a new method for mitotic chromosome spread preparation by which mitotic chromosomes were discharge and spread on the slide. This method was named the germ tube burst method (GTBM) by Taga and Murata (1994).

Fluorescence microscopy with different fluorescent dyes was introduced to the cytogenetics of filamentous fungi (Lemke *et al.* 1970, Raju 1986, Taga and Murata 1994). While meiotic preparations using acriflavine introduce a modest improvement, GTBM has been proven to be a powerful technique for mitotic cytogenetics in filamentous fungi in combination with DAPI staining (Taga *et al.* 1998, 1999, 2003, Akamatsu *et al.* 1999, Tsuchiya and Taga 2001a, Chuma *et al.* 2003). So far, visualization of mini-chromosomes is limited to GTBM using DAPI staining (Taga *et al.* 1999, Chuma *et al.* 2003, Mehrabi *et al.* 2007, Garmaroodi and Taga 2007).

Another break through is the introduction of fluorescence *in situ* hybridization (FISH) to the filamentous fungi. FISH technique was introduced in 1980. Instead of using isotopes in the previous ISH, this technique utilizes fluorochromes, several improvement introduced by the new technique include the ability to detect multiple targets using multiple colors simultaneously and safety. Although it had been applied widely in plant and animal cytogenetics, it is firstly applied in fungi by Taga and Murata in 1994 to detect rDNA. Subsequently, it was successfully applied for identifying 1.6 Mb conditionally dispensable chromosome in *N. haematococca* MPI (Taga *et al.* 1999, Garmaroodi

and Taga 2007), rDNA condensation (Taga *et al.* 2003) and fiber FISH (Tsuchiya and Taga 2001b, Tsuchiya *et al.* 2002).

A major revolutionary point for analyzing fungal chromosomes was the application of pulsed felid gel electrophoresis (PFGE). PFGE was first applied to *Saccharomyces* (Schwartz and Cantor 1984, Carle and Olson 1985) and since then the name "molecular" or "electrophoretic" karyotype was introduced. The electrophoretic karyotype displays the whole chromosome set as bands. Each band supposedly corresponds to a chromosome separated on an agarose gel by migration rate in an electric field with alternating poles. Chromosomes number and the sizes can be measured in kilobases (kbs) or megabases (Mbs). This technique has advantages in accurate measurement of chromosome size, detection of minute chromosomes, and analysis of karyotype polymorphisms, but has limitations in the resolution of large chromosomes (> 8-10 Mb) as well as co-migrating chromosomes with equal size (Beadle *et al.* 2003), and also yields no morphological information. PFGE had not only revealed the intraspecies chromosome polymorphisms in fungi (Mills and McCluskey 1990, Zolan 1995), but also helped in the discovery of B chromosomes, or nonessential, supernumerary chromosomes which was first recognized among the fungi by Miao *et al.* (1991) and subsequently the identification of the supernumerary and conditionally dispensable chromosomes (for review see Covert 1998).

Nectria haematococca mating population I

Nectria haematococca mating population I (MPI) is the causal pathogen of the Fusarium crown, foot and fruit rot of cucurbits. The name of this fungus has been changed from time to time. As for its teleomorph, *Hypomyces ipomoeae* (Snyder and Hansen 1940), *H. solani* f. *cucurbitae* (Hansen and Snyder 1943), *H. solani* f. sp. *cucurbitae* (Snyder *et al.* 1975) and *N. haematococca* var. *cucurbitae* (Bistis and Georgopolus 1979) have been recorded in the literature. Currently, *N.*

haematococca is the widely accepted scientific name (Booth 1971) with the reservation that there is an opinion that *Hematonectria* should be used instead of *Nectria* as the genus name (Rossman *et al.* 1999). As for its anamorph stage, it was first recorded as *F. javanicum* v. *theobromae* (Doidge and Kresfelder 1932). Then, *Fusarium solani* v. *cucurbitae* was erected for this fungus without discrimination of races (Snyder and Hansen 1941). In 1961 race 1 and race 2 were separated in this taxon based on the difference in pathogenicity (Tousson and Snyder 1961), leading to the current name of *F. solani* f. sp. *cucurbitae* races 1. On the other hand, *N. haematococca* is known to comprise mating populations, each probably corresponding to a biological species (Matuo and Snyder 1973, VanEtten and Kistler 1988). With regard to *F. solani* f. sp. *cucurbitae* race 1(Fsc1), it belongs to mating population I (MPI) among MPs I to VII, numbered according to date of discovery (Matuo and Snyder 1973) and most recently as FSSC 10 (O'Donnell 2000, Zhang *et al.* 2006).

Fusarium crown, root and fruit rot was first reported on squash (*Cucurbita pepo* L.) in 1930 in South Africa and subsequently reported in United States, Europe, Australia, New Zealand, Japan and recently in many different geographical regions. It can develop as a cortical rot of the crown and roots of the plant. Sporodochia form at the soil line on the infected plant, and macroconidia from sporodochia then infest the surrounding soil. In soil, conidia of Fsc1often form chlamydospores, which can survive for several years. Chlamydospores and conidia germinate to infect the fruit rind in contact with moist soil, causing fruit infections characterized by dry rot and sporodochia formation. After Fsc1 penetrates the fruit rind and then the flesh, it grows into the seed cavity. Once in the seed cavity, the fungus penetrates the seed and remains between the seed coat and the cotyledons but does not invade the cotyledons or reduce seed viability (Tousson and Snyder 1961, Snyder *et al.* 1975).

Also, *N. haematococca* MPI is heterothallic and produces perithecia readily by artificial crossing in the laboratory and eight unordered ascospores are formed in a linear array in the ascus and at maturity the ascospores ooze from the ostiole of each perithecium (Georgepoulos 1963, Snyder *et al.* 1975). In 1940s Snyder and his colleagues pioneered conventional genetics of this fungus, in which traits relating to sexual reproduction such as mating type and perithecial color was analyzed (Snyder and Hansen 1940, Hansen and Snyder 1943, 1946). Subsequently, other traits including cultural appearance, heterothallism and tolerance to fungistatic compounds were studied (for reviews, see Snyder *et al.* 1975, VanEtten and Kistler 1988). Although such genetic studies on *N. haematococca* MPI were ahead of genetic analysis of other plant pathogenic fungi in those days, investigations ceased after 1970. As to the karyotype of *N. haematococca* MPI, cytological investigations were done more than 50 years ago, in which haploid chromosome number (CN) of this fungus was estimated to be n=4 (Hirsch 1947, 1949, El-Ani 1954, 1956). Thereafter, no additional data have been added to the cytological karyotype of this fungus. This situation is in marked contrast to those of model fungi such as *N. crassa* and *Saccharomyces cerevisiae*, in which their comprehensive karyotypes were established by different methodologies including PFGE, conventional light microscopy, electron microscopy and genetic linkage analysis (Zolan 1995).

Objectives of the current study

The objective of this study was to establish the method of chromosome preparation and determine the karyotype of *N. haematococca* MPI. Besides the conventional meiotic cytology, karyotyping was carried out by combining the two recent methods used for fungal karyotype, PFGE and GTBM. Through this study, a new mini-chromosome represented in one strain of this fungus was analyzed by PFGE, cytological visualization, FISH, random amplification of polymorphic

DNA (RAPD) and genetically to study its mode of inheritance through meiosis using both random and unordered ascospores analysis. This study will contribute in the karyotype evolution of *Nectria-Fusarium* species complex study project.

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CHAPTER II

MOLECULAR KARYOTYPING BY PULSED FIELD GEL ELECTROPHORESIS

SUMMARY

Molecular karyotyping was attempted for the two standard strains ATCC18098 and ATCC18099 by PFGE. With running conditions to separate chromosomes up to ~ 6 Mb, two middle-sized chromosome bands were clearly resolved but larger chromosomes were not separable. These two bands migrated similarly between the two strains and their sizes were estimated to be ~ 3.5 and ~ 2.5 Mb. Besides these bands, an extra small band was detected in ATCC18098 with a size of ~ 410 kb. Southern hybridization of this band with telomere repeats (TTAGGG)n from *N. crassa* as a probe showed the presence of telemetric repeats within this band suggesting that this band is a mini-chromosome rather than a huge plasmid. Large chromosomes were not separated even though further attempts were made. EKs of the two strains remained unknown in this study.

INTRODUCTION

The characteristics of the genome in terms of the number of chromosomes and the microscopic morphology of each of chromosome are called karyotype. In plants, the information provided by karyotype analysis is often used as a taxonomical trait in species description and in genetics for studying chromosome number variations and morphological aberrations. In fungi, karyotyping by cytological methods is much more difficult because the chromosomes are extremely small and mitotic division arose within the nuclear membrane. Therefore, the technique has not been applied extensively. The karyotyping using chromosomes in meiotic cells and in post meiotic mitosis was feasible and

chromosomes count had been obtained for several fungi (for review see Wieloch 2006). Studying chromosome morphology by meiotic karyotyping had not been very successful and this approach is not possible in asexual species.

The introduction of PFGE has allowed the separation of chromosome-sized DNAs. Thus "electrophoretic" or "molecular" karyotypes can be obtained relatively easily. The electrophoretic karyotype, which displays the whole chromosome set, consists of bands, each corresponding to a chromosome separated on an agarose gel by migration rate in an electric field with alternating poles. This can provide a count of the chromosomes number and the sizes of the chromosomes measured in kilobases (kbs) or megabases (Mbs). Pulsed field gel electrophoresis (PFGE) was first applied to *Saccharomyces* (Schwartz and Cantor 1984, Carle and Olson 1985) and since then it has been applied for the filamentous fungi. This technique has advantages in accurate measurement of chromosome size, detection of mini-chromosomes, and analysis of karyotype polymorphisms. But it has limitations in the resolution of large chromosomes (~ 8 Mb) as well as co-migrating chromosomes with equal size (Beadle *et al.* 2003) and it also yields no morphological information.

To date, for many fungal species of all major classes, PFGE has been applied to generate valuable information on variation in chromosome size and number within and between species, on genetic linkage of markers as well as applications in gene mapping and cloning and in analyzing transformants and chromosome mutations (for review, see Olsen 1989, Mills and McCluskey 1990, Walz 1995). Curiously, the electrophoretic karyotyped (hereafter, called EKs) of many other *N. haematococca* including MPII, MPIII, MPIV, MPVI and homothallic strains have been published (Miao *et al.* 1991, Nazareth and Bruschi 1994, Taga *et al.* 1998, Suga *et al.* 2002), Whereas that of MPI has not been reported. Thus, presently available information of the karyotype of MPI is

only from classical cytology (Hirsh 1947, 1949, El-Ani 1954, 1956). This study is the first report about the EK of *N. haematococca* MPI

MATERIALS AND METHODS

Fungal strains and culture

Two standard strains of *N. haematococca* MPI, ATCC18098 and ATCC18099, purchased from American Type Cultural Collection, were used in this study. Maryland. They were recovered on synthetic low nutrient agar (SNA: 1 g KH_2PO_4, 1 g KNO_3, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.2 g glucose, 0.2 g sucrose, 20 g agar, adjusted to 1 L using distilled water)(Nirenberg 1990) 4ºC. Potato dextrose agar medium (PDA, 200 g Potato infusion, 10 g Dextrose, 20 g Agar adjusted to 1 L using distilled water) was used for routine culture and potato dextrose broth (PDB, 200 g Potato infusion, 10 g Glucose, adjusted to 1 L using distilled water) was used for production of budding cells (microconidia).

Protoplast isolation

Protoplasts were prepared from germinated budding cells as described by Taga *et al.* (1998). Budding cells were produced by inoculating 40 ml of PDB in 100 ml conical flask with several pieces of mycelia agarose blocks cut from slant cultures, incubated by rotary shaking at 200 rpm in 20°C. Budding cells from 1-week-old culture were harvested in sterile condition and filtered through one layer of Kimwipe to remove hyphal debris. The conidial suspension was added to 100 ml PDB in 300 ml flask at a final concentration of 1×10^6 conidia ml^{-1} and incubated on a reciprocal shaker (100 strokes min^{-1}) overnight at 27°C. The germlings were vacuum-filtrated on a Whatman no. 4 filter paper, washed with 0.8 M $MgSO_4$, then suspended in 5 ml filter- sterilized enzyme solution [25 mg of Driselase (Kyowa Hakko), 5 mg of Cellulase Onozuka RS (Yakult Pharmaceuticals), and 5 mg of Kitalase (Wako Pure Chemicals) dissolved in 1 ml

of 0.8 M $MgSO_4$] and incubated on a shaker (70 strokes min^{-1}) for 3 to 4 h at 30°C. Resulting protoplasts were filtered through six layers of Kimwipe, collected by adding 5ml of 0.8 M NaCl solution and centrifugation at 1000 ×*g* for 15 min. After carefully decant the supernatant solution, pelleted protoplasts were washed twice by the later solution by centrifugation.

Preparation of agarose plugs

Protoplast-agarose plugs were prepared according to the method of Miao *et al.* (1991) with some modifications. Briefly, the pellet of protoplasts was suspended in SE (1 M sorbitol, 50 mM EDTA, pH 8.0) at a concentration of $4–6 \times 10^8$ /ml, mixed with an equal volume of molten 1% (w/v) low-melting-point agarose (Bio-Rad,) dissolved in SE, and solidified in the molds to make plugs. The plugs were soaked in NDS [0.5 M EDTA, 10 mM Tris-HCl, pH 8.0, 1% (w/v) *N*-lauroylsarcosine sodium salt] at 37°C for at least 14 h. After three washes of 30 min each in 50 mM EDTA (pH 8.0), the plugs were stored in the same solution at 4°C until use.

Pulsed field gel electrophoresis

A contour-clamped homogenous electric field-type apparatus (CHEF DRII, Bio-Rad) was used for chromosome separation. For the separation of chromosomes smaller that ~ 6Mb, 0.8% agarose gels (pulsed field certified or chromosome grade agarose, Bio-Rad) was used. The running buffer was 0.5× Tris-borate-EDTA (Sambrook *et al.* 1989), which was kept at 10°C during the runs. 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, a 1,800 to 1,300 s interval for 36 h at the same voltage, 1.8 V/cm with a 1300 to 800 s interval for 42 h, and 2.4 V/cm with a 800 to 600 s interval for 36 h. For chromosomal DNAs larger than 6 Mb, running condition was: 0.55% agarose, 35 V with ramped pulse time from 4500 to 13000 s for 360 h. For separation of small chromosomal DNAs, running

condition was: 0.8% agarose; 200V, 60s, 12 h followed by 90 s, 8 h using same voltage. As size marker, chromosomal DNAs of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* purchased from Bio-Rad were used as size markers,.

After finishing electrophoresis, whole or the desired lanes of the gels were stained with ethidium bromide (0.5 μg/ml) for 1 h, destined in water for 1 h, and then photographed under UV illumination. ImagJ (http://rsb.info.nih.gov/ij/) was used for calculating the intensity of the resulted band

Southern blot hybridization

Membrane preparation

Chromosomal DNAs from PFGE gel were alkaline-transferred to Hybond N+ nylon membrane (Amersham Biosciences, Bucks, UK). Following electrophoresis and staining, gel was visualized on a UV-illuminator and photographed; the gel was then trimmed for the desired lanes and removed the unused areas. The gel was soaked in three gel volumes of depurination buffer (0.25 N HCl) for 30 min at room temperature with gentle agitation, rinsed with distilled H_2O, then soaked in denaturation buffer (0.4 N NaOH/1.5 M NaCl) for 30 min (15 min each) with gentle shaking. Using a vacuum blotting apparatus (Atto, Tokyo, Jaapn), a piece of Hybond N+ (Amersham) membrane cut slightly larger the gel and pre-wet in distilled H_2O was placed on middle of the upper side of the porous supporter. The whole surface including outer edges was covered with plastic film excluding an area slightly smaller and on the top of the used membrane. The gel was then placed on the top of the membrane. Avoiding any air trapping, the upper supporter was firmly tightened. The gel was covered with the transfer buffer (0.25 N NaOH) which is periodically poured onto the gel during transfer. After chDNA transferred using 0.01 MPa for 1.5 h, apparatus was disassembled and the membrane was rinsed twice with 2× SSC for 15 min with gentle shaking,

marked for the desired lanes, dried at 80°C for 10 min, and kept in dehydrated condition until use (Sambrook *et al.* 1989).

Probe preparation

Plasmid pNC36 (Schechtman 1990) containing telomere repeats (TTAGGG)n of *Neurospora crassa* was used for E. coli BH5α competent cells transformation. 200 µl of competent cells were mixed with 10 ng of plasmid DNA and incubated on ice for 30 min. Subsequently, cells were subjected to a heat shock for 30 seconds at 42°C water bath, then been returned to ice for 5 min followed by adding 1 ml of SOC medium (20 g of Bacto tryptone, 5 g of Bacto yeast extract, 2 ml of 5 M NaC, 2.5 ml of 1 M KCl, 10 ml of 1 M $MgCl_2$, 10 ml of 1 M $MgSO_4$, 20 ml of 1 M glucose, Adjust to 1 liter with distilled H_2O). After 1 h shaking incubation at 37°C (200 stroke/mint), 10-50 µl of the cells were plated on Luria broth (LB) -ampicillin plates (LB medium: 5 g NaCl, 5 g tryptone, 2.5 g yeast extract, 10 g agar in 500 ml distilled H_2O. After autoclaving the medium and let cool to 55°C, 500 µl of 100 mg/ml filter-sterilized ampicillin were added then poured into Petri dishes. After solidification, 100µl X-gal were spread on agar surface just prior inoculation). After overnight incubation at 37°C, positive colonies were selected individually and disperse in 50 ml of LB-ampicillin media in a 500 ml flask. The cultures were incubated at 37 °C with vigorous shaking for 2 h then each 40 ml of this culture were transferred to a 50 ml conical tube and centrifuge at 3000 x g for 10 min. Pellets were subjected for extraction and purification of plasmid using alkaline lysis based Quantum prep plasmid midiprep kit (Bio-Rad) according to the manufacturer's manual.

Plasmid was subjected to overnight digestion using *Hin*dIII, (0.5µl enzyme was added to 3-5 µl plasmid solution, 1 µl buffer in a total of 10 µl reaction mixture) at 37°C followed by agarose gel separation of the resulted fragments (1.2% agarose in 1x TBE buffer, 100 V for 30 min). Gel was stained in 0.5 µg/ml ethidium bromide solution and the band visualized using UV illumination. The ~

750-bp fragment was cut from the gel and subjected for DNA extraction using QiaexII gel extraction kit (Qiagen) according to the manufacturer's instruction. After measuring the resulted DNA concentration with spectrophotometer it was kept in -20°C until used.

For probe labeling, hybridization and detection, the AlkPhos Direct Labeling and Detection System (Amersham Biosciences) was used according to the manufacturer's instruction. Signal detection was made on a X-ray film (Fuji Medical X-ray film).

RESULTS

Protoplasting of fungal cells and preparing of agarose blocks is a key step in preparation of intact chromosomal DNA. I optimizing a reliable condition for obtaining large amount of good quality protoplasts with relatively shorter time and low coast for the two strains had been tested in this study. First, I standardized the conditions related to the source of protoplast such as the type of mycelia used for production of conidia, conidia preparation culture, type and age of conidia and the age of mycelia used for protoplasting. Overnight germinated freshly produced budding cells were used for protoplasting. From the results of each single enzyme and some enzymes combinations tested (Table II. 1). Driselase proofs its superiority than other cell wall degradation enzymes in releasing protoplasts, by adding kitalase and cellulose to the previous enzyme in low concentrations improves the number of released protoplasts (2-4 X 10^7 per ml of enzyme mixture). Also, such combination is more efficient using MgSO4 as an osmic-regulator solvent solution then NaCl. Homologous shaped protoplasts of different size were obtained (Fig. II. 1). The quality of the prepared protoplasts was tested after preparation of the protoplast-embedded agarose blocks with PFGE by using the specified concentration (~4–6 X 10^8 /m).

The electrophoretic karyotyping of ATCC18098 and ATCC18099 was attempted using three standard conditions for resolving chromosomes size larger than 6.0 Mb, chromosomes size from 6.0 Mb to 0.5 Mb, and chromosome size of less than 2.0 Mb. With a standard running condition of PFGE suitable to separate chromosomes ranging from 6.0 Mb to 0.5 Mb, complete separation of chromosomes using such conditions was not achieved. Although large chromosomes were not separated and clumped in one band in the upper side of the gel, two similar bands are resolved: the first band was ca. 2.5 Mb and the second band ca. 3.5 Mb in each strain. Also, a single small-sized band was resolved in strain ATCC18098 (Fig. II. 2).

Since co-migration and clumping of bands hampered complete chromosome resolution in both strains under the running conditions mentioned above, attempts were made to resolve all chromosomal DNAs into discrete single bands using strain ATCC18098. For such purpose, the gel of lower agarose concentration was used in combination with low electrical voltage and longer ramping time as reported in the previous literatures (Orbach *et al.* 1988, 1996, Brody and Carbon 1989, Debets *et al.* 1990, Eusebio-Cope *et al.* 2009). Using 0.55% agarose gel concentration and the specified running conditions suitable for resolving large chromosomes, one faint band which might represent the largest chromosome was resolved in the uppermost of the gel. The size of the band was estimated to be ca. 8 Mb compared with the largest chDNA of S. *pombe* and assuming the linear correlation between size and immigration distance. The other large chromosomes were not resolved, forming one broad band. The mid-sized two bands were separated with apparently wider sizes than those in the previous run, which may suggest that each band corresponded to more than one chromosome. The smallest band was resolved as a very thin stained band (Fig. II. 3). For testing the possibility the two resolved bands were doublet, a relatively short run was designed using the same agarose condition of the first run (1.5 V/cm, ramping pulse 3,600 s to 1,800 s, 72 h). The two medium-sized bands were

clearly resolved in this gel using these conditions and each band was intensely stained by ethidium bromide, again suggesting the possibility that each of these two bands represented doublet chromosomes. Beside the smallest band resolved in the previous gels which appeared in sharp staining, two faint staining wider bands were evident (Fig. II. 4), thus raising the question about the nature of these small bands.

Using running conditions suitable for separation of small chromosomes, one small band was resolved clearly, whereas the other two smaller bands that were resolved in the previous PFGE experiment turned to a faint smear (Fig. II. 5A). Southern hybridization using the telomere repeats (TTAGGG)n from *N. crassa* as a probe showed the presence of telemetric repeats within this band (Fig. II. 5B). From the result of southern hybridization as well as the constant detection of this small band under different running conditions, this small band was thought to be a mini-chromosome rather than a giant plasmid.

To calculate the size of this mini-chromosome accurately, relative migration distance of the chDNA of *S. cerevisiae* from three different electrophoretic runs using the previous running conditions were calculated and plotted against the size of the corresponding band (Table II. 2). Assuming proportional relationship between size and migration distance and by calculating migration distance of the small band in the three experiments, the size of this band was estimated to be ~ 410 kb.

DISUSSION

In fungi, protoplasts can be defined as spherical cells whose chitinous cell walls have been removed by appropriate digestive enzymes (Bachmann and Bonner 1959). With respect to hyphal age, results vary from fungus to fungus but in general, 18–48 h old mycelia, corresponding to the early to mid-exponential growth phase result in the best protoplast yield (Peberdy 1979). Based on the

early observations on protoplast formation in Fusarium species (Lopez-Belmonte *et al.*. 1966), the hyphal walls swell at specific points before release of the protoplasts. Presumably these points are the initial sites of the enzyme attack and are most accessible when the hyphae are actively growing. Making use of such information, I used young hyphae resulting from the germination of freshly harvested budding cells.

For protoplasting, different enzymes and enzyme combinations were used in publications for protoplasting of other *F. solani* strains. For example, Drislase 3% (Ha *et al.* 1991), lysing enzyme 2.7mg/ml containing cellulose, protease and chitinase (Nazareth and Bruschi, 1994), a combination of four enzymes, Novozym 234, Cellulase Onozuka-RS, β-glucuronidase (type H1) and Zymolyase-20T (Taga *et al.* 1998), a combination of six enzymes, Novozym 234, Cellulose Onozuka RS, Zymolyase 20T, β-glucuronidase type H1, Driselase and Chitinase (Suga *et al.* 2002) were used. The incubation times in those publications were ranged from 2-4 h. In this study, incubation time was in the same range, but combination of drislase (25 mg/ml) and both cellulase Onozuka RS and kitalase (5 mg/ml) at a a relatively low concentration was used effectively for producing protoplast of good quality. This is a simple and low-coast protocol that maybe introduced to other fungal strains for obtaining protoplasts for chDNA preparation or protoplast fusion experiments.

Even PFGE has replaced cytology in routine karyotyping of fungi and widely used for many fungi (for reviews see, Mills and McCluskey 1990, Walz 1995). There are two limitations in EK using PFGE. Firstly, if two or more chromosomes are of similar size, they migrate in a similar distance on the gel to form co-migrant band. Secondly, if the chromosomes of the entire complement range over quite a large size difference, so the whole chromosomes set cannot be resolving using single running conditions (Fincham *et al.*. 1979). Unfortunately, *N. haematococca* MPI has both limitations: two resolved bands co-migrated

doublet which could be identified by their staining intensity and even different running condition were used the large chromosomes clumped in one broad band. Similarly, the EKs of *Fusarium* species have been already published including some strains belonging to other mating populations MPII to MPVII except for MPV (Nazareth and Bruschi 1994, Taga *et al.* 1998, Suga *et al.* 2002), even each strain had its own EK pattern, co-migrated chromosomes, and chromosomal bands larger than 6 Mb were reported in these publications.

PFGE has been applied for resolving large chDNAs of many fungi such as *N. crassa* (Orbach *et al.* 1988), *Aspergillus* spp. (Brody and Carbon 1989, Debets *et al.* 1990), *F. solani* (Nazareth and Bruschi 1994) and *Manaporthe grisea* (Orbach *et al.* 1996). For resolving large chromosomes, running conditions were adapted using stepwise separation. In such procedures, many running trials using lower agarose concentration combined with lower voltage, long ramping time and prolonged running time were applied. To correspond the resolved large band to chromosomes, restriction fragments or chromosomes-specific markers should be used. For *N. haematococca* MPI, neither genetic linkage map nor physical map that represents individual chromosomes to serve as the source of chromosome-specific markers is available. Therefore, identification of individual chromosomes in the clumped band as has been done for *N. crassa* (Orbach *et al.* 1988) and *M. grisea* (Orbach *et al.* 1996) was difficult for this fungus. The absence of EK for this fungus in past studies may be explained similarly.

As an alternative method for assessing the chromosomes number is telomere fingerprint analysis. In this method, genomic DNA was first digested by restriction enzymes with no cutting site in the telomere repeat sequence arrays. Then the electrophoretically separated bands were probed with labeled telomere sequences. Ideally, the number of bands hybridized with the telomere probe represents double the chromosomes number. This method was applied successfully with some fungal strains to assess chromosomes number (Sanchez-

Alonso *et al.* 1996, Viaud *et al.* 1996, Levis *et al.* 1997, Tadanori 2002, Farman and Yun-Sik 2005, Eusebio-Cope *et al.* 2009). In this study, I abandoned this method because it would only give an unreliable estimation of the chromosomes number affected by the co-migration of two or more telomere fragments. On the other hand, I used the telomere probe to confirm the nature of the small band resolved under the different running conditions. Identification of linear plasmid from fungi has already been reported for many species (Akins *et al.* 1986, Cullen *et al.* 1985, Francou *et al.*., 1987, Garber *et al.*., 1984, 1986, Francis and Michelmore 1993) including *N. haematococca* MPI (Samac and Leong 1988). However, the small band in the strain ATTC 18098 was proven by southern hybridization to include fungal telomere sequences of (TTAGGG)n (Schechtman 1990, Coleman *et al.* 1993). From this result, the band was judged to be a mini-chromosome (Beadle *et al.* 2003, Covert 1998, Mils and McCluskey 1990).

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Table II.1. Cell wall degrading enzymes tested for releasing protoplast cells from *N. haematococca* MPI.

Enzyme	Source organism	Supplier	Concentration (mg/ml)
Driselase	*Basidiomycetes*	Kyowa Hakko	5-25
Glucanex	*Trichoderma sp.*	Novo Nordisk	5-25
Lysing enzyme	*Trichoderma harzianum*	Sigma	5-25
Kitalase	*Rhizoctonia solani*	Wako Pure Chemicals	5-10
Cellulase Onozuka RS	*Trichoderma viride*	Yakult Pharmaceuticals	5-10

Table II.2. Calculation of the size of the min-chromosome. Data representing the immigration distance of chromosomal DNA bands of *Saccharomyces cerevisiae* used for standard curve construction between size and migration distance.

Chromosomal DNA band	Migration distance		
S. cerevisiae	Exp. 1	Exp. 2	Exp. 3
610	596.00	630.80	481.87
565	620.40	655.20	516.80
450	679.20	706.00	585.60
365	723.60	750.40	649.07
285	767.60	792.00	699.21
Mini-chromosome	698.80	726.40	611.47
Calculated size	399.8	415.3	417.2

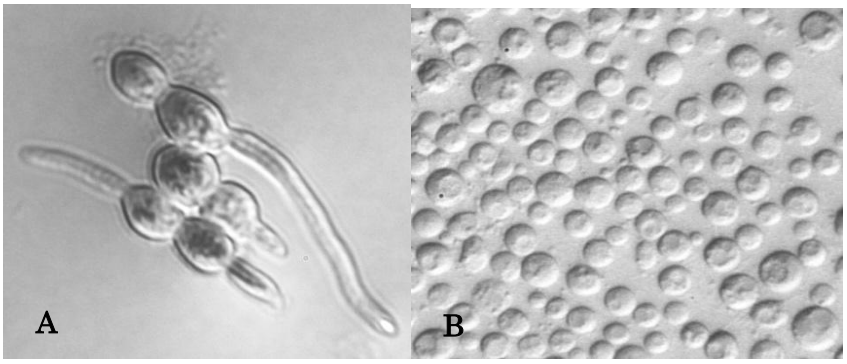

Figure II. 1. A. Germination of budding cells, B. Protoplasts of *N. haematococca* mating population I of various sizes.

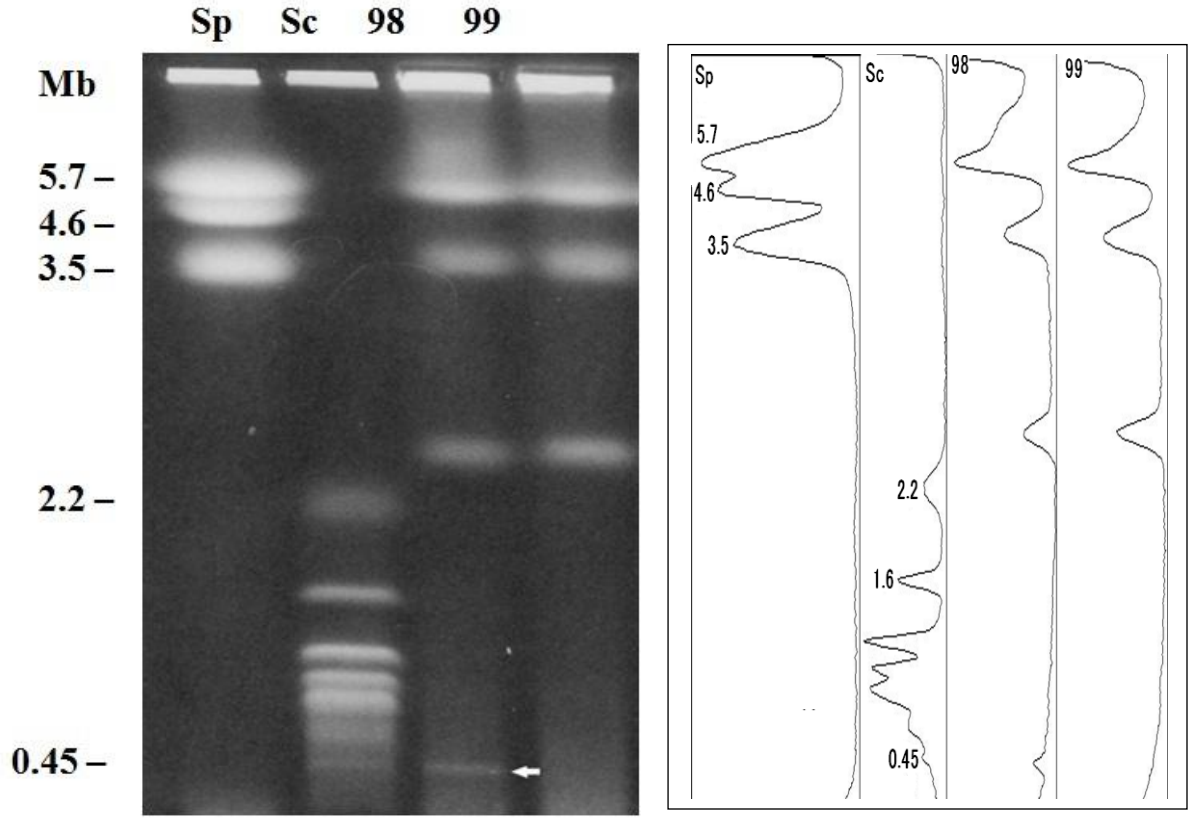

Figure II. 2. Separation of chromosomal DNAs below ~6 Mb and the represented lanes plot. Running conditions: 0.8% agarose, 50 V, ramped pulse time from 3,600 to 1,800 s, 115 h; 50 V, from 1,800 to 1,300 s, 24 h; 60 V, from 1,300 to 800 s, 24 h; 80 V, from 800 to 600 s, 27 h. Sp: *Schizosaccharomyces pombe*. Sc: *Saccharomyces cerevisiae*. 98: ATCC18098. 99: ATCC18099. Numbers to the left of each panel indicate DNA sizes.

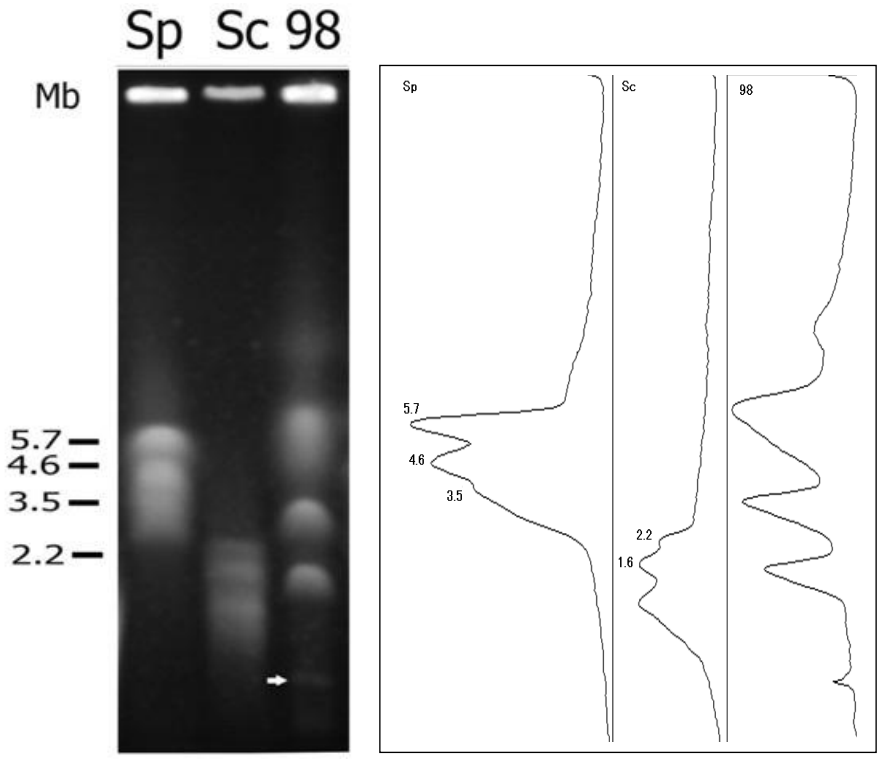

Figure II. 3. Modified condition for separation of large chromosomal DNAs of strain ATCC18098 and the represented lanes plot. Running conditions: 0.55% agarose, 35 V, ramped pulse time from 4500 to 13000 s, 360 h. Sp: *Schizosaccharomyces pombe*. Sc: *Saccharomyces cerevisiae*. 98: ATCC18098. 99: ATCC18099. Numbers to the left of each panel indicate DNA sizes.

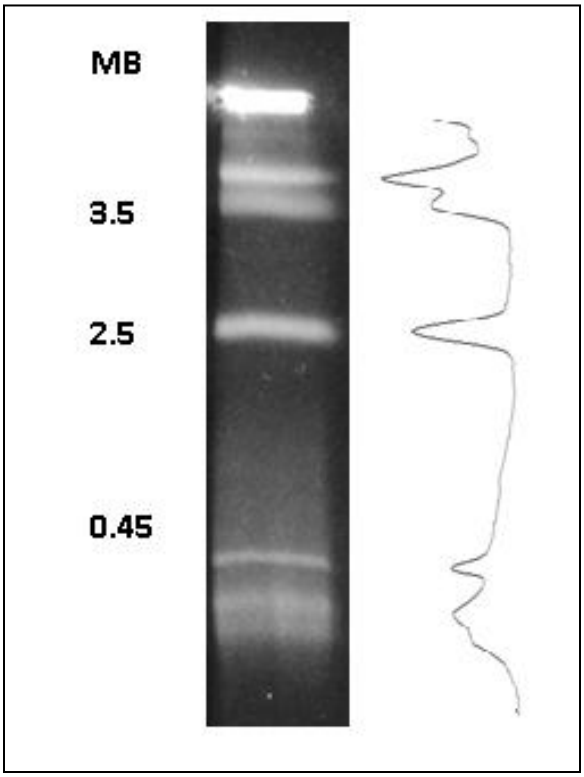

Figure II. 4. Separation of middle size chromosomal DNAs of strain ATCC 18098 and the represented lane plot. Conditions were modified as follows: 0.8% agarose, 50 V, ramped pulse time from 3600 to 1800 s, 72 h. Numbers indicate the relative DNA size in Mb.

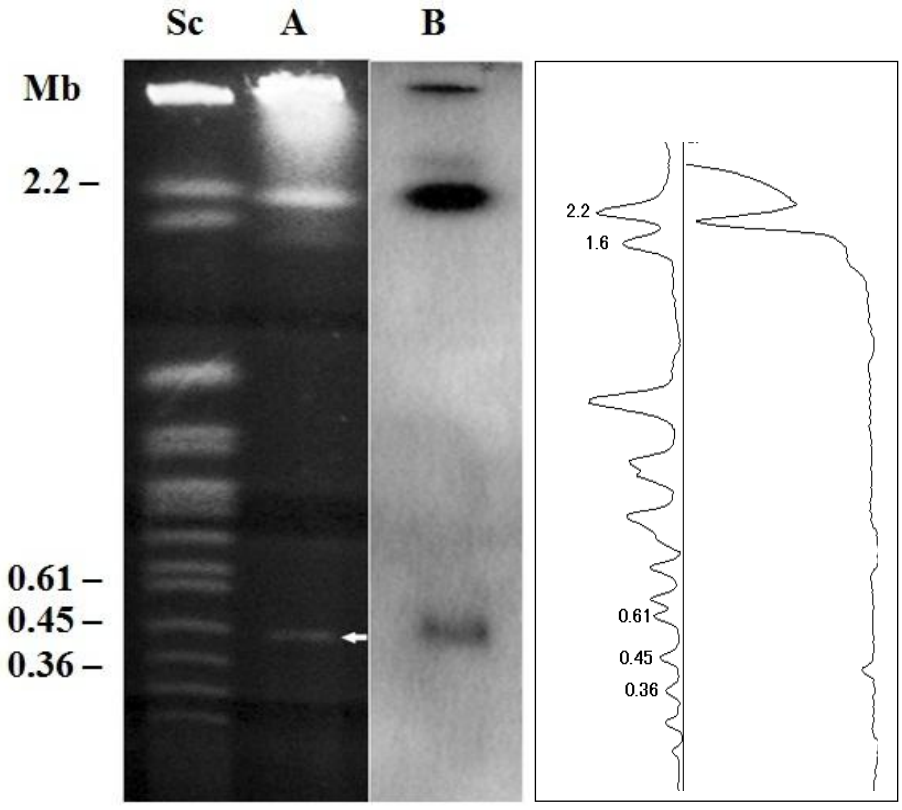

Figure II. 5. A. Separation of small chromosomal DNAs of strain ATCC 18098 and the represented lanes plot. Running conditions: 200 V, 60 s, 12 h; 90 s, 8 h. B. Related southern hybridization analysis with a telomere probe. Sc: *Saccharomyces cerevisiae*. Numbers to the left of each panel indicate DNA sizes.

CHAPTER III

CYTOLOGICAL KARYOTYPING USING MEIOTIC CHROMOSOME PREPARATION

SUMMARY

By employing DAPI staining method, meiotic and post-meiotic nuclear divisions in the asci derived from the crossing between strain ATCC 18098 and strain ATCC 18099 were studied. A total of four nuclear divisions took place during ascosporeogenesis in which the single diploid nucleus divided to eight haploid dinucleated ascospores within a single ascus. Chromosome count during pachytene was hampered by the aggregation of elongated chromosomes. Chromosomes in the subsequent stages of meiosis I were more or less discretely separated but accurate count was not confirmed and reliable karyotyping on such stages seemed difficult due to the clumping of some chromosomes. Chromosomes in meiosis II or post-meiotic mitosis were not suitable either for the same reason.

INTRODUCTION

With *N. crassa*, McClintock (1945) developed the fixing and staining methods for describing the meiotic cycle and the following mitotic divisions in the ascus and she was able to identify each of the seven *Neurospora* chromosomes at pachytene by a combination of size, chromomere pattern and, for chromosome 2, the nucleolus attachment. The centromere position could be identified by the shape of the chromosomes at first mitotic prophase or anaphase, assuming that the bend in a chromosome with a V or L shape indicated centromere position.

Using smear preparations stained with hematoxylin mixture, iron-acetocarmine or acetic lactmoid, Hirsch (1947, 1949) pioneered the cytological investigations of *N. haematococca* f. *cucurbitae*. On the basis of her observations, she concluded that the haploid CN for this fungus is n=4 in the hermaphrodite, n=3 in the male, n=3 in the female and n=2 in the neuter. Later, El-Ani (1954, 1956) studied the nuclear behavior, especially during diakinesis, pro-metaphase I and metaphase I of the same fungus using acetoorcien smear technique and concluded that the CN in each of the four strains he examined n=4 thus correcting the previous CN count. Howson *et al.* (1963) concluded that CN of this fungus either 4 or 5 assuming that it has more than one basic chromosome number.

Recently, Taga *et al.* (1998) reported 6 and 7 chromosomes for a homothallic strain of *N. haematococca* using acetoorcien and iron-hematoxylin preparations where the latter staining agent was used successfully as in *Neurospora* (Raju and Newmeyer 1977; Raju 1978). Even such chromosome count was in harmony with the previous chromosomes count of n=6 (Hirsch 1949) and n= 5 (El-Ani 1954, Howson *et al.* 1963), mitotic CN for the same strain was n=12-13. Thus concluded the meiotic preparations underestimated the chromosomes number for this fungus.

On the other hand, the DNA-specific fluorochromes such as acriflavine and DAPI were suggested to be used for meiotic visualization (Raju 1982). Acriflavine had been used successfully for detailed meiotic chromosome analysis of *Neurospora* instead of the conventional staining methods (Raju 1986, Perkins *et al.* 1995). Moreover, DAPI (4′,6-diamidino-2-phenylindole), another flourchrome stain was applied successfully in mitotic chromosomes visualization of fungi (Taga and Murata 1994) but failed to stain the meiotic chromosomes of *Pyricularia oryzae* (Nirmala 1999), In this study, I tried to use DAPI for meiotic

karyotyping with relatively simple procedures to visualize chromosome behavior during meiotic division and reach a reliable chromosomes count for this fungus.

MATERIALS AND METHODS

Fungal strains and crossing

Two standard strains of *N. haematococca* MPI, ATCC18098 and ATCC18099 were used in this study. ATCC18098 is a MAT1-1 hermaphrodite that produces red perithecia as a female and ATCC18099 is a MAT1-2 hermaphrodite with white perithecia. Crossing between ATCC18098 and ATCC18099 were set up by the modified procedure of VanEtten (1978). Conidia of each strain were separately spread on V8 juice agar (M-29, Stevens 1974) in plastic Petri dishes (10-cm diameter) and mycelia were allowed to develop by incubating at 22–24°C for 10–15 days under continuous fluorescence lighting. For spermatization, about 10 mL of conidial suspension prepared from the PDA plate culture was poured on the mycelia of the strain acting as the female. After 5 min, the conidial suspension was drained, and the fertilized cultures were incubated as above. Pre-mature perithecia for meiotic observation were harvested after 7-10 days.

Meiotic chromosomes preparation

Following the procedure suggested by Raju (1982), perithecia containing developing asci were placed on a glass slide in a drop of 25% (v/v) glycerol solution and gently crushed with a hand-made flexible glass needle under a stereo microscope to release asci. After removing the perithecial walls and any debris, 15 µl of 1 µg/mL DAPI dissolved in antifade mounting solution (Johnson and Nocueira Araujo 1981) was added and the samples gently covered with a coverglass without pressing. Observation was made immediately by UV-excitation using an epiflourescence microscope (Olympus BH2/BHS-RFC)

equipped with a 100× oil immersion objective lens (N.A. = 1.3). Fluorescent images were recorded using a digital camera (Olympus C5050-Z) attached to the microscope and processed with Adobe Photoshop ver. 10.0.

RESULTS

Although the present investigation was mainly confined to the study of karyotype of this fungus, brief observations were also made on the stages of nuclear fusion and subsequent divisions in the ascus.

A total of four nuclear divisions were recognized during ascosporeogenesis of this fungus. Two meiotic divisions, in which meiotic division I (M-I) resulted in the division of a single diploid nucleus into two haploid nuclei and meiotic division II (M-II) yielded four haploid nuclei (Fig. III. 1). A third mitosis gave rise to eight haploid nuclei (Fig. III. 2A-D), which were delimited into either four biseriate or eight uniseriate ascospores. The fourth division takes place inside these ascospores and their nuclei give rise to an ascus containing eight binucleated ascospores (Fig. III. 2E-J).

The stages prior to meiosis in which cells with condensed diploid chromosomes were prepared to start meiosis were difficult to be visualized. At meiosis I, different stages were hardly distinguished due to the few numbers of cells during this division. At prophase I, chromosomes were decondensed to form thin threads but still some condensed regions could be visualized at early leptotene (Fig. III. 1A). These chromosomes became fully uncondensed and paired at diplotene and zygotene. At pachytene, brightly stained and elongated chromosomes, which were probably paired chromosomes, were evident with many intercalary highly stained knobs. Chromosomes count was not clear due to their overlapping (Fig. III. 1B). At diakinesis, chromosomes started their condensation again and stained segments were more clearly observed in this stage with cross-linked fine strands. At the end of this stage and with the full

condensation of the chromosomes, metaphase I starts (Fig. III. 1D). Chromosomes of metaphase I were highly condensed. It was clear from the degree of staining and size that each of these chromosome bodies represented a bivalent chromosome. Each bivalent disjoined towards an opposite pole at anaphase I and the number of chromosomes was supposed to be doublet (Fig. III. 1E, F). In both stages, chromosomes aggregation and overlapping hindered accurate chromosome counting. Haploid CN in these stages ranged from 4-6. It is worth mentioning that the frequency of metaphase I nuclei in the specimens was very low comparing to anaphase I nuclei, suggesting that the division proceeded quickly through metaphase I.

Starting from division II (Fig. III. 1G-K), stages preceded quickly with slight difference in the rate of division between the two sister nuclei. Within some specimens, delay of apical nuclei led to the presence of different stages at one time in the same ascus (Fig. III. 1H-J). Also, an ascus with three nuclei was observed but it was easily to judge that one nucleus was doublet from its size (Fig. III. 1I). Chromosomes during these stages were highly condensed but still aggregated. 4-5 chromosomes could be counted during early metaphase II and early anaphase II in some specimens. Even though there was asynchrony of the division of the two nuclei during the earlier phases of meiosis II, four nuclei were commonly observed in late telophase II (Fig III. 1K).

During the third division, time lagging was less evident among nuclei. Within some asci, the two middle nuclei started their division but still the other two apical and basal nuclei were at interphase (Fig. III. 2A). Such asynchrony could be recognized within the following stages with an apical nucleus at prophase III, two middle nuclei at metaphase III and basal nucleus at early metaphase III. For chromosomes count, four chromosomes could be distinguished at metaphase III (Fig. III. 2B). Generally, nuclei preceded to the next division stages in a relatively short time. The resulting eight nuclei at the

end of this division were oriented in a biseriate (Fig. III. 2D) or in a uniseriate (Fig. III. 2E) order, thus resulting in four binucleated or eight uninucleated haploid ascospores within the ascus, respectively.

In mitotic fourth division, which occurred inside the haploid ascospores (4 binucleated or 8 uninucleated), the nuclei were more clearly stained with DAPI at this division. Nuclei at prophase IV were large with many highly stained small dot like chromatin regions (Fig. III. 2E). These highly stained regions were more clearly recognized in the following metaphase (Fig. III. 2F). Although, chromosomes count was not clear at this stage due to the overlapping of the chromosomes, a relatively long thin protrusion which may be related to the rDNA or the nucleolus organizing region (NOR) was visualized clearly. Chromosomes at anaphase IV chromosomes were dot like shaped and condensed (Fig. III. 2G). Count of these chromosomes was not clear because of the background shadows of the stain but at least seven highly stained dots were countable in one nucleus. Unlike the nuclei at the telophase III, only binucleated ascospores were recognized at the end of telophase IV (Fig. III. 2H) and finally, eight haploid two-celled ascospores within each ascus arose (Fig III. 2I-J).

DISCUSSION

Observations on the nuclear divisions in asci on *N. heamatococca* have been carried out with conventional staining using either hematoxylin (Hirsch 1949, Taga *et al.* 1998) or acetoorcien (El-Ani 1954, Howson *et al.* 1963, Taga *et al.* 1998). Such conventional staining methods provided clear definition of chromosomes morphology at pachytene in a number of fungi. Beside the requirement of exceptional preparations and skills, these methods are time consuming and give variable results (Barry 1966, 1996, Lu and Raju 1970, Raju and Newmeyer 1977), so simple and reliable methods for visualization of the meiotic division in fungi are needed. Both fluorochromes, DAPI (Coleman and Goff 1985) and acriflavine (Raju 1986) worked satisfactory for staining nuclei in

asci. Acriflavine staining was much favored than DAPI staining due to the prolonged time of observation without fading (Raju 1982), but such staining involved long preparatory procedures such as hydrolysis. With the recent availability of antifade reagents (Johnson and Nocueira Araujo 1981), DAPI staining with its specificity to AT-rich regions is much easier and applicable to meiotic chromosomes visualization in asci than acriflavine. Although DAPI does stain neither the nucleolus nor spindle as with iron-hematoxylin, unlike the previous report about the failure of DAPI to stain the sexual stages of *Pyricularia oryzae* (Nirmala 1999), DAPI was successfully applied to this fungus and most of meiotic stages could be visualized and photographed with satisfactory resolution.

Meiotic stages of the crossing between the two strains ATCC18098 and ATCC18099 were examined in this investigation. Although detail observation of chromosomes was not achieved satisfactorily due to both the small size and the aggregation of chromosomes, results showed that this fungus proceeded normally during meiosis, and four successive divisions could be visualized (digitally edited in Figure III. 3). Both the time lag and asynchrony during meiosis II and diagonal spindle poles were reported for other fungi including *N. haematococca* (for review see Snyder *et al.* 1975, VanEtten and Kistler 1988, Zickler 2006). Asynchrony of nuclei may result in some asci with abnormal ascospore number of less than eight, and diagonal orientation of chromosomes during meiotic metaphases leads to unordered tetrads.

Meiotic chromosomes in pachytene have been routinely used for karyotyping in conventional cytology for fungi (see Lu 1996) and for this fungus as well (Hirsch 1949, El-Ani 1954). They confirmed the presence of four bivalent chromosomes in this stage: one large NOR-chromosome, two medium and one small sized chromosome. In this study, pachytene was characterized by elongated bright chromosomes with terminal and intercalary highly stained

knobs. These bivalents were found unsuitable for karyotyping because of their aggregation. Knobs were intensely stained with DAPI, and hence supposedly AT-rich heterochromatin.In addition, NOR-chromosome could not be identified in this stage. Compared to pachytene, chromosomes in the subsequent stages of meiosis I were more or less discretely separated but accurate count was not possible. Also, chromosomes counting in the post meiotic mitosis were not so accurate because of the tiny size of the chromosomes in these stages and also chromosomes tended to aggregate. From all the visualized stages, chromosome numbers were either 4 or 5. The clear aggregation of the chromosomes during meiosis suggests that this count is not reliable.

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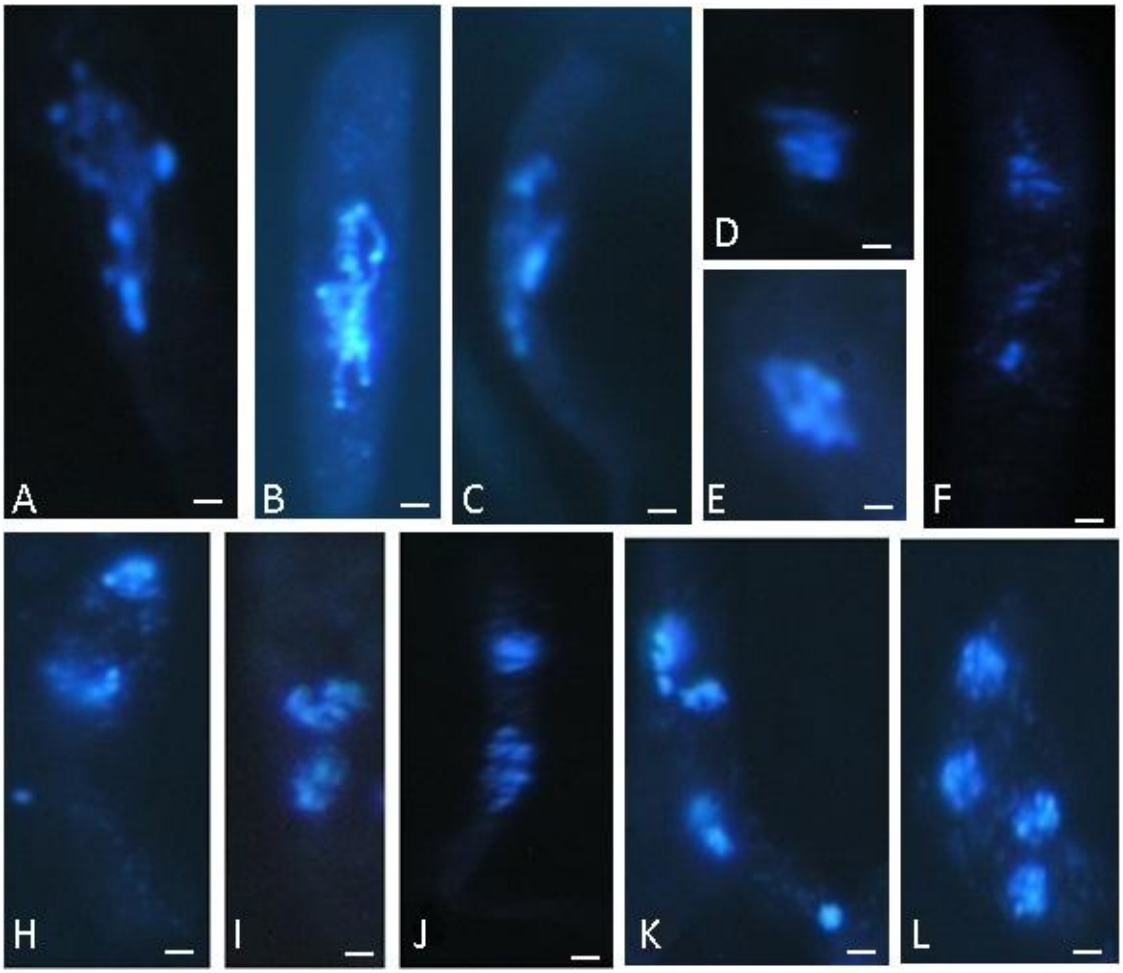

Fig. III. 1. Meiotic chromosomes visualization of the crossing of stain ATCC 18098 x ATCC 18099. Meiosis I (A- F), A. Zygotene chromosomes de-condensate to form thin strands, some regions still in the process of de-condensation. B. Pachytene thin overlapped chromosomes with highly stained intercalary knobs are evident, C. Early diaknasis, chromosomes start to re-condensate and appeared as short highly condensed parts crossed with thin strands. D. Early metaphase I, chromosomes are fully condensate forming dot-like highly stained bodies aggregated in the equator of an ascus. E. Early anaphase, ideally, each chromosome set moves toward opposite pole, spindle was not stained but can be suggested from the direction of the chromosomes movement. F. Late anaphase I, two sets of chromosomes are evident, 4 highly stained bodies could be visualized which may reflect the chromosomes number of this fungus. Meiosis II (G-K), G. Interphase nuclei, 3-4 highly stained dot shaped bodies could be distinguished. H. One nucleus at prophase (basal) and the apical nucleus at metaphase, I. Basal nucleus divided at anaphase but still the apical nucleus at metaphase, J. Ascus with three nuclei, the lower two resulted from telophase, the apical one still at anaphase, 7-8 chromosomes of the apical nucleus could be counted. K. Four nuclei at the end of Meiosis II.

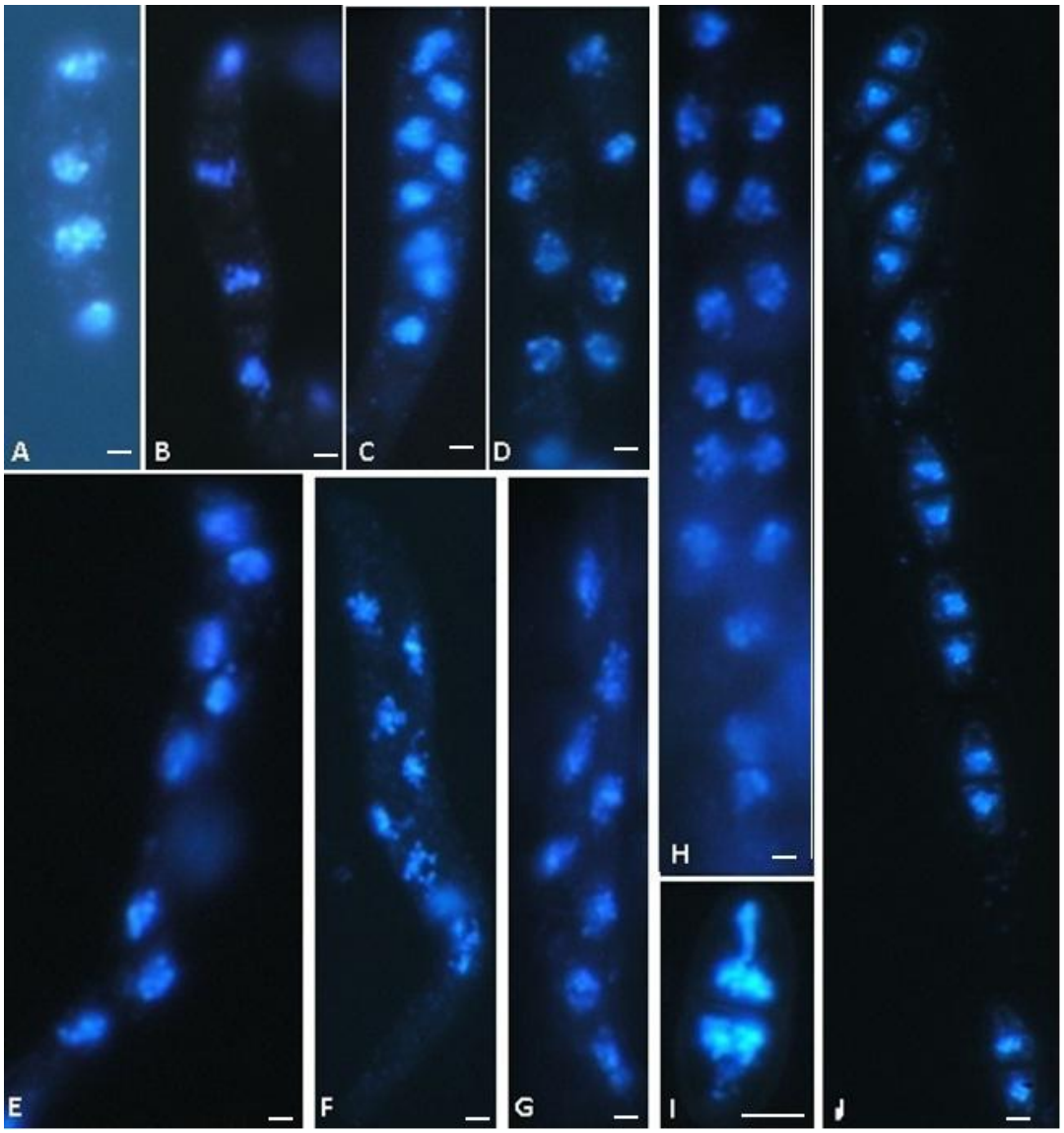

Fig. III. 2. Post meiotic mitosis. Nuclear division III (A-D). A. Four nuclei at prophase with apparent highly stained dot like regions. B. Phase lag in one ascus, the four asynchronized nuclei, apical nucleus is out of focus in this image, the two middle nuclei are at metaphase, the basal nucleus is at early metaphase, 4 chromosomes could be counted in the metaphase nuclei. C. Eight nuclei at telophase III, D. Four biseriate ascospores at the end of telophase. Nuclear division IV (E-J). E. Eight uniseriate ascospores at early prophase, four nuclei have clear heterochromatin dot like structures with one of them is located outside the nucleus. F. Chromosomes at metaphase, some chromosomes are thread like with intensely stained knobs, the thin long protrusion maybe related to NOR-chromosome in some asci. G. Anaphase IV, dot-like chromosomes start to separate. H. Asci at the end of telophase IV, each of the 8 asci is binucleated. I. An ascus with eight haploid dinucleated ascospores, J. Single dinucleated ascospores.

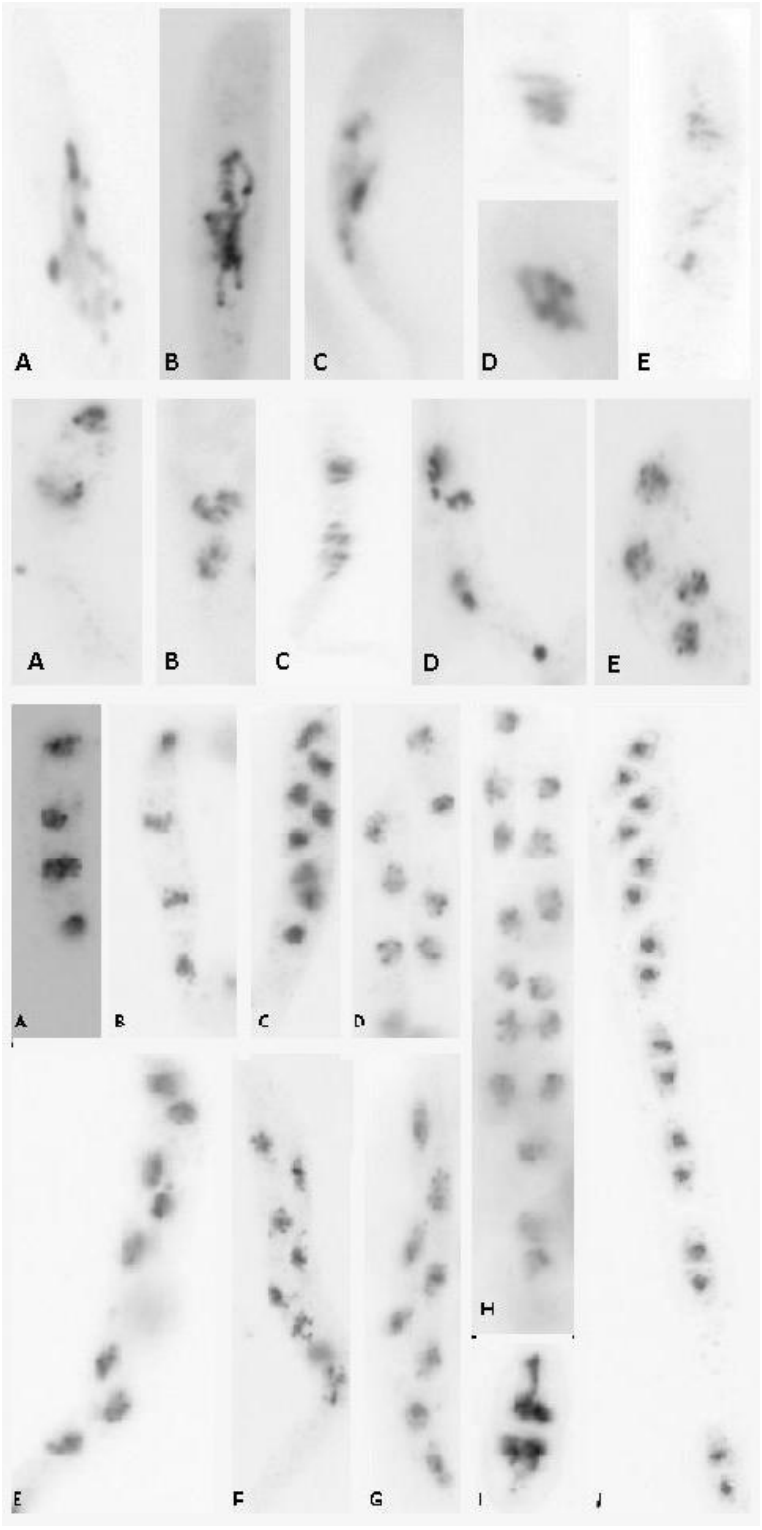

Fig. III. 3. Digitally inverted monochrome of the four divisions.

CHAPTER IV

CYTOLOGICAL KARYOTYPING BY THE GERM TUBE BURST METHOD

SUMARRY

Cytological karyotypes with mitotic metaphase chromosomes were analyzed by the germ tube burst method (GTBM). Prior to karyotyping, procedures of GTBM suitable to *N. haematococca* MPI were established by examining conditions for sample preparation and staining including treatment for arresting metaphase and for the staining reagents. In both ATCC18098 and ATCC18099, nine chromosomes were routinely counted with an extra mini-chromosome in ATCC18098, thus correcting the previous chromosome count by conventional meiotic cytology. Detailed karyotyping for each strain was obtained using chromosome length and other features such as intensely fluorescing segments (IFSs) and the protrusion of rDNA. Comparing the two karyotypes, relative ratios of chromosome length among the complements were somewhat similar between the two strains, whereas chromosomes were not homologous between the two strains by judging from the difference in IFS. Chromosome 5 in each strain had a rDNA protrusion at its end, thus correcting the previous report that rDNA was associated with the longest chromosome. In addition, the 410-kb mini-chromosome discovered in this study is the third smallest mini-chromosome cytologically visualized in filamentous fungi.

INTRODUCTION

Preliminary data of the karyotype of *N. haematococca* MPI have been obtained by conventional light microscopy on the meiosis. Hirsh (1949) concluded that the CN in a hermaphrodite was four including one large, two medium and one small

chromosomes. She also suggested that both male and female strains had three chromosomes and the neuter only two chromosomes. On the other hand, El-Ani (1954) rejected such suggestion based on his genetic investigation on the same fungus. Later on, he showed that the CN is four (El-Ani 1956). Howson (1963) suggested the presence of more than one CN for this fungus. Recently, it is our common view that the reliability of data obtained using convention meiotic preparations is considered to be doubtful. The germ tube burst method (GTBM, Shirane *et al.* 1988, Taga and Murata 1994) enabled visualization of mitotic chromosomes of conidia-producing fungi, with which condensed chromosomes discharged from the hyphal cell and spread on the surface of slides gave better resolution and reliable count than before. This method showed that the previous conventional cytology is often erroneous in counting meiotic chromosomes (Taga *et al.* 1998, Tsuchiya and Taga 2001) and such data need to be revised. In this study, cytological karyotyping was attempted using GTBM for obtaining a reliable karyotype of this important plant pathogen.

MATERIALA AND METHODS

Fungal strains and culture

Two standard strains of *N. haematococca* MPI, ATCC18098 and ATCC18099, were used in this study. PDA was used for routine culturing. Budding cells were produced as described in Chapter II. For producing macroconidia, 40 ml of mung bean broth (Gale *et al.* 2005) in a 100-ml Erlenmeyer flask was inoculated with mycelia agar plugs, followed by shaking on a rotary shaker for 3–4 days at 20–25 C. The conidial suspension was filtered through one layer of Kimwipe to remove mycelia debris and the conidia were washed twice with water by centrifugation. Finally, conidia were suspended in PDB at a concentration of ~4×10^5/ml.

Preparation of chromosome specimens

Two methods, GTBM and the dropping method were used for preparing chromosomal specimens, the latter was newly developed in this study.

In the GTBM, to prepare germlings that adhered to a slide, a drop of 100-150 µl conidial suspension was placed in a square (24 × 32 mm) lined with paper cement on a poly-L-lysine-coated clean slide, and incubated at 25°C for 6–7 h in humid condition to allow germination. For arresting metaphase, PDB was replaced with 100 µl of fresh PDB containing 50 µg/ml thiabendazole (TBZ). After an additional incubation was resumed for 1 h in the same condition, slides were dipped in a staining jar containing water for ~30 s to wash off the broth and the excessive water on the slide was removed with filter paper. The slides were gently immersed in a fixative solution (methanol:acetic acid 17:3) for 30 min at room temperature then flame-dried and stored at room temperature until use.

In the dropping method, germinated budding cells prepared by gentle overnight shaking in PDB were harvested and washed twice in distilled water by centrifugation. The pelleted germlings were suspended in ice cold fixative (methanol:acetic acid=3:1) for more than 1 h. One hundred to 150 µl of the suspension was gently dropped on the center of a slide preheated at 80°C, and immediately transferred to a hot, humid condition made with a water bath kept at 60°C. Slides were then air-dried and stored until use.

Fluorescence microscopy

Chromosome specimens were mounted in antifade solution (Johnson and Araujo 1981) containing DAPI (1 µg/ml) or both DAPI (1 µg/ml) and propidum iodide (PI, 0.5 µg/ml), covered with cover glasses and sealed with nail polish. Chromosome observation was made by UV-excitation using an epiflourescence microscope (Olympus BH2/BHS-RFC) equipped with a 100× oil immersion objective lens (N.A. = 1.3). Fluorescent images were recorded using a digital camera (Olympus

C5050-Z) attached to the microscope and processed with Adobe Photoshop ver. 10.

RESULTS

Preparation of specimens

Prior to karyotyping, methods to visualize mitotic chromosomes were optimized for *N. haematococca* MPI using ATCC18098. For specimens' preparation, both GTBM and dropping method developed in this study were used. Staining dye, DAPI only or double staining with DAPI and PI (DAPI/PI) was tested. Also, conditions for metaphase nucleus arrest using treatment with TBZ were tested.

In dropping method, specimens' preparation was easier with fewer preparative steps than GTBM. Chromosome spreads of good quality were obtained when dropping distance was 25–30 cm above the slide. Most of the chromosome preparations were in prophase and chromosomes were rather elongated and stacked together (Fig. IV. 1A, B). The metaphase chromosomes specimens prepared with GTBM were more suitable for karyotyping in terms of the characterization of morphological features of chromosomes. However, the frequency of obtaining metaphase samples was lower, and some chromosomes tended to stick to each other to hinder chromosome counting (Fig. IV. 1C, D).

As for staining, DAPI/PI staining highlighted chromosome regions that were differentially stained with these two dyes (Fig. IV. 1). For comparison of different mitotic arresting conditions, metaphase frequency was relatively high and fully condensed chromosomes were discretely separated to enable reliable chromosome counting in GTBM with 1 h treatment with TBZ (Fig. IV. 2). Consequently, we decided to use GTBM combined with TBZ treatment and DAPI/PI staining for karyotyping (Fig. IV. 1F).

Karyotype

In both ATCC18098 and ATCC18099, nine chromosomes were routinely counted with an extra mini-chromosome in ATCC18098. Thus, CNs of ATCC18098 and ATCC18099 were determined to be n=10 and n=9, respectively. Detailed karyotyping for each strain was attempted with the representative specimens (Fig. IV. 3). Chromosomes were first aligned in the order of chromosome size measured in longitudinal axis length, and numbered in the decreasing order of size. Then, features of each chromosome such as intensely fluorescing segments (IFSs) and the protrusion of rDNA were picked up by visual judgement, and finally all the data were integrated into idiograms. The reproducibility of IFS pattern and protrusion of rDNA among samples was confirmed in each strain using two additional specimens selected from different slides.

In ATCC18098, distinct features usable for the instant identification of specific chromosomes were: intercalary IFS in chromosome 2, terminal IFS in chromosome 3, protrusion of rDNA in chromosome 5, and intercalary IFS in chromosome 7. The other chromosomes besides chromosomes 2, 3, 5 and 7 were also identifiable based on the combination of IFS and chromosome length as illustrated in the idiogram. Among the chromosome complements, chromosome 5 was designated as NOR chromosome because rDNA is referred to as nucleolur organizer region (NOR) in terms of its function. Although chromosome 10 was too small (ca. 0.4–0.6 μm in length) to recognize IFS inside, the whole chromosome was stained more with DAPI than with PI, suggesting that this chromosome is relatively AT-rich.

In ATCC18099, distinct features as the markers for rapid identification of specific chromosomes were: intercalary IFS in chromosome 3, terminal IFS in chromosome 4, protrusion of rDNA in chromosome 5, and intercalary IFS in chromosome 7. Chromosomes 1, 2 and 6 were identifiable from the others by the distribution patterns of IFS combined with chromosome length. Regarding

chromosomes 8 and 9, they could be distinguished from the rest in that they were the smallest and did not have IFS, but distinction between these two chromosomes was difficult.

Comparing the two karyotypes, chromosome 1 of each strain and chromosomes 3 and 7 of ATCC18098 and chromosomes 4 and 7 of ATCC18099 seemed to be homologous as indicated by the idiograms (Fig. IV. 4). In contrast, the other chromosomes were not homologous by judging from the difference in IFS. Typically, chromosome 5 in each strain had a rDNA protrusion at its end, but the two chromosomes were different to each other in the occurrence of intercalary IFS. Putting aside IFS, relative ratio of chromosome length among the complements were somewhat similar between the two strains except chromosome 5.

DISCUSSION

In this study, CNs of two strains *N. haematococca* MPI were determined to be n=10 for ATCC18098 and n=9 for ATCC18099 by mitotic cytology with GTBM. These CNs are in marked contrast to the previous estimate of n=4 for this fungus that was obtained by conventional meiotic cytology (Hirsch 1947, 1949, El-Ani 1954, 1956). Although variation in CN is known to occur even among strains in the same fungal species (for review see Zolan 1995), this great discrepancy in CN between the two types of cytology is not reasonably explainable just by the difference of strains used in this and previous studies. Considering the high quality of our specimens and the proven reliability of GTBM for chromosome counting (Taga *et al.* 1998, Akamatsu *et al.* 1999, Tsuchyia and Taga 2001, Eusebio-Cope *et al.* 2009), this estimate is thought to represent correct CN for this fungus.

Compared to PFGE, cytology has merits in that it does not have a size limitation for chromosomes and in that it is feasible to discover morphological

characters chromosomes. Such merits of cytology have been well exemplified in performing mitotic cytology with GTBM for *F. gramineanum* whose genome consists of four large chromosomes exceeding 7 Mb (Gale *et al.* 2005) and also for *C. parasitica* that contains five similar-sized chromosomes in its genome (Eusebio-Cope *et al.* 2009). This study presents an additional example of the value of cytology for fungal karyotyping. While cytological information is meaningful in its own, it is also useful in complementing PFGE. For instance, the 3.5 Mb and 2.5 Mb chromosomal bands separated by PFGE in both ATCC18098 and ATCC18099 were uncertain as to their state as singlets or doublets. With the result of cytology that both strains have two pairs of small, similar-sized chromosomes, that is, chromosomes 6 and 7 as one pair and chromosome 8 and 9 as another pair, each of these two bands is deducible to be doublet. Similarly, an estimate of the genome size of this fungus can be calculated which is ~ 40Mb (Figure IV. 5).

In cytological karyotyping, IFS and rDNA served as the useful morphological markers for the identification of chromosomes. Regarding IFS, four chromosomes out of nine were identified in each strain by noticing intercalary or terminal IFS. IFS was first used for cytological karyotyping in *C. parasitica* and regarded as AT-rich constitutive heterochromatin by actinomycin D/DAPI staining (Eusebio-Cope *et al.* 2009). An intriguing finding about IFS in this study was that distribution of IFS on the chromosomes was significantly different between ATCC18098 and ATCC18099. Obviously, chromosome rearrangements could be a potent cause of such difference since they occur ubiquitously among strains in filamentous fungi (Kistler and Miao 1992, Zolan 1995). However, the signifcance difference in the number of IFS between the two strains, i. e. 20 in ATCC18098 except 410-kb mini-chromosome vs. 10 in ATCC18099, seems rather difficult to explain solely by the chromosome rearrangements such as deletion or duplication.

As to rDNA, it appeared as a thread-like protrusion from the apex of chromosome 5 in both strains, and hence, chromosome 5 was designated as NOR chromosome. This observation is not compatible with the report of El-Ani (1956) that the NOR chromosome was the largest in the genome. The morphological feature of rDNA in the shape of its protrusion is not unique to *N. haematococca* MPI. A similar appearance of rDNA has been observed in GTBM-prepared chromosome specimens of other fungi and its entity as rDNA or NOR was proven by FISH (Shirane *et al.* 1988, Taga and Murata 1994, Taga *et al.* 1998, Akamatsu *et al.* 1999, Taga *et al.* 2003). Interestingly, NOR chromosomes of *N. haematococca* MPVI and homothallic *N. haematococca* were also the fourth or fifth largest among 12 to 17 chromosome complements (Taga *et al.* 1998). Comparing the karyotypes among *N. haematococca* MPVI, MPI and homothallic strains, each species has its own karyotype regarding the number of chromosomes, chromosomes identity and banding pattern. This indicates the importance of similar studies for the other mating populations to understand the karyotype evolution in *N. haematococca* species complex.

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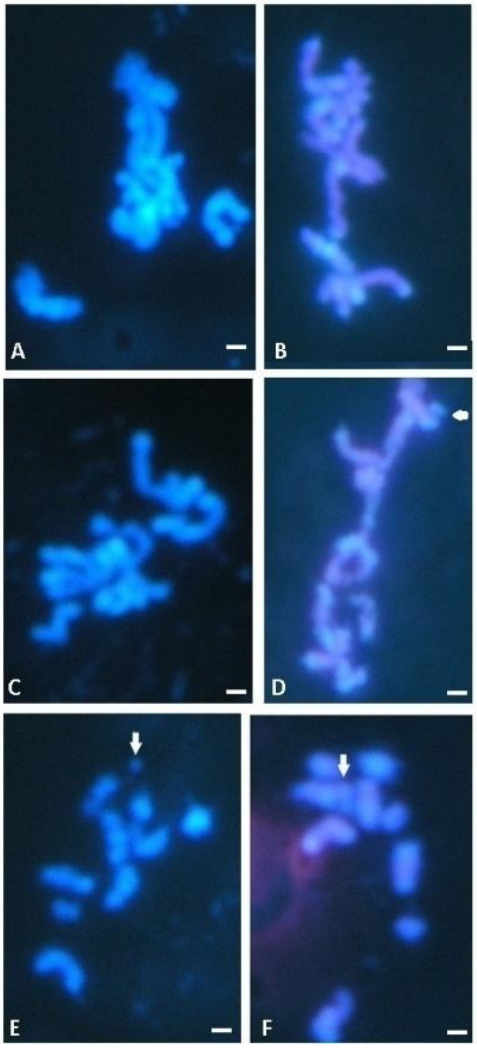

Fig. IV. 1. Mitotic chromosomes of *Nectria haematococca* MPI visualized by different preparation and staining methods. A-B. Metaphase chromosomes prepared by the dropping method stained with DAPI (A) or DAPI/PI (B). C-D. Metaphase spreads prepared by the germ tube burst method (GTBM) without thiabendazole (TBZ) treatment stained with either DAPI only (C) or DAPI/PI (D). E. DAPI-stained chromosomes in mid-metaphase prepared by GTBM combined with TBZ treatment. F. DAPI/PI-stained chromosomes in mid-metaphase prepared by GTBM combined with TBZ treatment, arrows indicate mini-chromosome Bars: 1 μm.

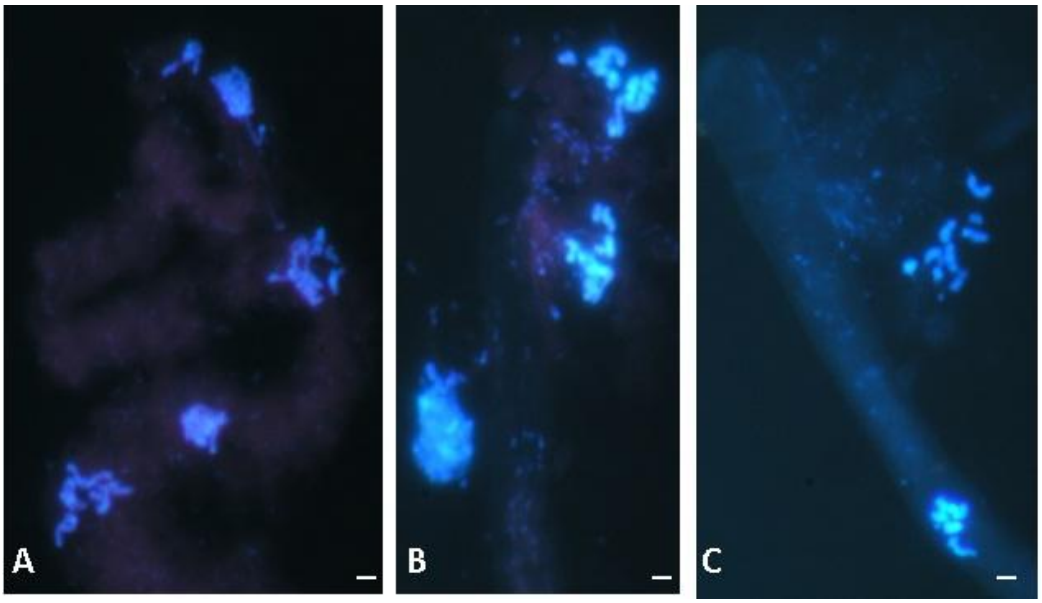

Fig. IV. 2. Arresting of the metaphase chromosomes of *N. haematococca* MPI during mitosis by GTBM using 50 μg/ml thiabendazole (TBZ). A. mitotic chromosome preparation without TBZ treatment, nuclei at different stages. B. chromosome sets after 30 min treatment using TBZ, nuclei at metaphase and prophase, chromosomes overlapping arose, C. Metaphase arrest with TBZ treatment for 1 h. Chromosomes discharged from the burst cell were relatively short comparing with those in B and no overlapped. Notice that the chromosomes remaining in hyphae were more condensed and aggregated than the discharged ones.

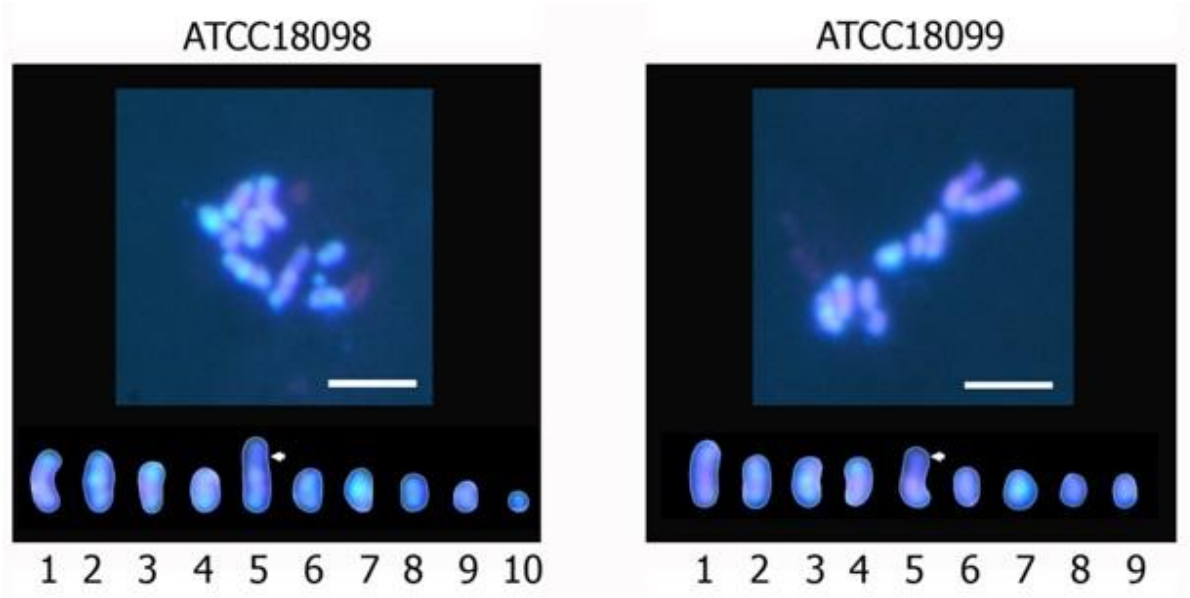

Fig. IV. 3. Cytological karyotypes of *Nectria haematococca* MPI strains ATCC18098 and ATCC18099 with mitotic metaphase chromosomes. Specimens were prepared by GTBM combined with thiabendazole (TBZ) treatment and stained with DAPI/PI. A. Chromosome spread of nucleus (upper in each panel) and chromosomes arranged by length (lower in each panel). Chromosome alignment was done by cutting each chromosome from the spread image, and arranging them in the decreasing order of axial length. The numbers 1 to 10 were assigned to the individual chromosomes. The arrowheads indicate rDNA protrusion on chromosome 5 in both strains. Bars: 2 μm.

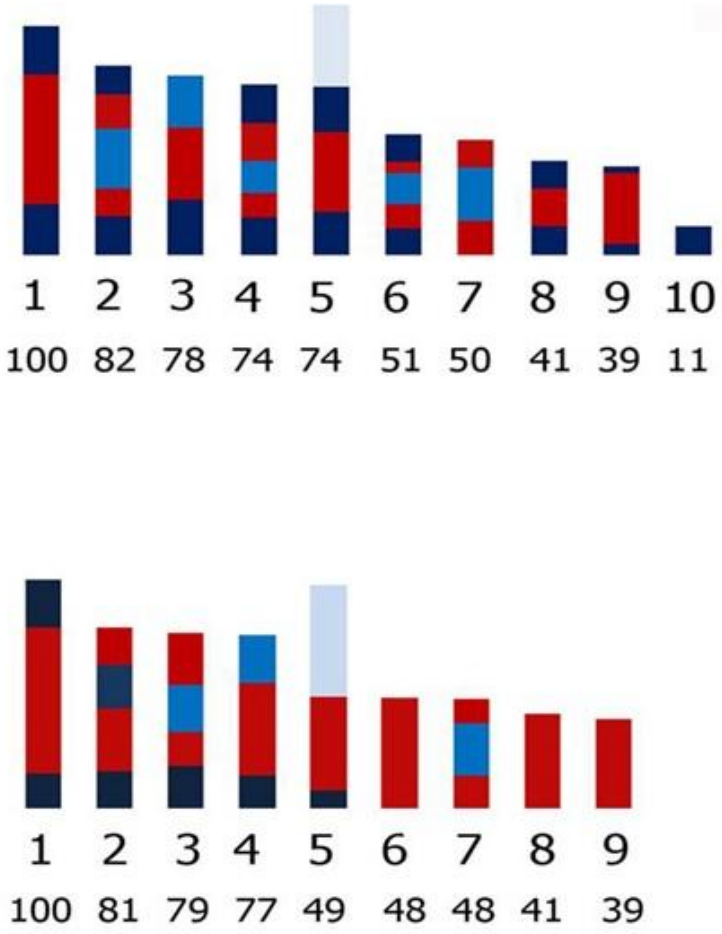

Fig. IV. 4. Idiograms corresponding to the aligned chromosomes in Fig IV. 3. The features of chromosomes were integrated into the idiograms. The areas painted with blue, light blue, and red represent intensely fluorescing segments, regions with relatively high affinity for DAPI, and regions with relatively high affinity for PI, respectively. The figures below the idiograms indicate relative percentage values of axial length of each spread.

CHAPTER V

CYTOLOGICAL ANALYSIS OF THE MINI-CHROMOSOME IN THE GENOME OF *NECTRIA HAEMATOCOCCA* MPI

SUMMARY

A small chromosome in the strain ATCC18098 was visualized by both GTBM and dropping method. The chromosome was minute of the size of ca. 0.6–0.8 μm in length and the whole chromosome was stained more with DAPI than with PI in GTBM preparations, suggesting that this chromosome is relatively AT-rich. In the specimens obtained by dropping method, this chromosome had a slender rod-like shape with terminal knobs on both ends during late prophase-early metaphase and was ca. 1.0–1.2 μm in length. Fluorescence *in situ* hybridization (FISH) using the 410-kb chromosomal DNA of strain ATCC18098 as a probe showed that the probe hybridized to the mini-chromosome in the mitotic specimen of strain ATCC18098 but not to any other chromosomes. Also, the probe hybridized to a single spot in the interphase nucleus of strain ATCC18098, proving that the 410-kb band is linked to DNA in the nucleus, not in the cytoplasm. In either case, hybridization signals were not detected elsewhere. The probe did not hybridize with the specimens of strain ATCC18099, either. These results confirmed that the 410-kb band on the PFGE gel represents the mini-chromosome detected in cytological observation and that it is not the derivative of other A chromosomes of either strains.

INTRODUCTION

B chromosomes have been described in more than 1,300 plant species and almost 500 animals (Camacho 2005). In fungi, molecular karyotyping with pulsed-field gel electrophoresis (PFGE) has identified B-like chromosomes in various species

of fungi which were mainly identified by their size (Mills and McCluskey 1990, Zolan 1995, Covert 1998). Unlike B chromosomes of plants and animals, the identification of mini-chromosomes in fungi has relied mainly on the visualization of DNA bands separated in agarose gels by PFGE. Such molecular visualization raises the concern that these DNA bands may be large linear plasmids rather than a mini-chromosome (Francis and Michelmore 1993). Either cytological visualization or demonstration of functional centromeric and telomeric regions is necessary to confirm that these DNA are chromosomes (Taga *et al.* 1999).

The name supernumerary or dispensable chromosome was introduced to describe the extra chromosomes composed primarily of DNA that is not present in at least one other, normally growing isolate of the species. As not all the mini-chromosomes are supernumerary (Chu *et al.* 1993, Mehrabi *et al.* 2007), southern hybridization, or recently FISH has been applied for distinguishing such chromosomes from ordinary A chromosomes. The supernumerary chromosomes carrying functional genes are referred as conditionally dispensable chromosomes (Covert 1998).

In this chapter, using both GTBM and dropping method, the cytological visualization of the mini-chromosome in the strain ATCC18098 of *N. haematococca* MPI was attempted to reveal its morphological feature. FISH technique was applied using the 410-kb chDNA band from PFGE as a probe to prove that this band corresponds to the mini-chromosome and examine whether it is a supernumerary chromosome or not.

MATERIALS AND METHODS

Fungal strains and culture

The two standard strains of *N. haematococca* MPI, ATCC18098 and ATCC18099 were grown on PDA for general purposes. Mung bean broth (Gale *et al.* 2005) and PDB were used for producing of macro- and microconidia, respectively.

Preparation of chromosome specimens

Cytological specimens of mitotic chromosomes were prepared either by GTBM or by the dropping method as described in chapter IV. For chromosome observation, specimens were stained with DAPI (1 µg/ml) or both DAPI (1 µg/ml) and PI (0.5 µg/ml) dissolved in the antifade mounting solution. Microscopic observations, photography and image processing were carried out by using the same system as used for meiotic observation in Chapter III. Measurements of axial length of chromosomes were made with ImageJ 1.44. http://rsbweb.nih.gov/ij/index.html).

Fluorescence *in situ* hybridization (FISH)

Chromosome painting FISH was carried out according to the method of Taga *et al.* (1999) using the 410-kb chDNA of ATCC18098 as a probe. To prepare probe DNA, protoplast agarose plugs were prepared and subjected to PFGE as described in Chapter II with some modifications. To avoid shearing of DNA during isolation from the band, only the left- and rightmost lanes were stained to visualize 410-kb band under UV illumination. The unstained part of the lanes corresponding to the position of 410-kb band was then excised and subjected to another run of PFGE to ensure purity of DNA. After finishing the second run, agarose blocks that contain the target chDNA band were cut from the gel as in the first run and DNA was extracted using GeneClean II kit (BIO 101). Finally, DNA was subjected to amplification with REPLI-g kit (Qiagen), followed by labeling with biotin-14-dATP by nick translation using a BioNick Labeling System (Invitrogen) according to the manufacturers' instructions. Prior to hybridization, specimens were treated with RNase (100 µg/ml) dissolved in 2x SSC (300 mM NaCl, 30 mM sodium citrate) at 37°C for 1 h, then rinsed in 2x SSC and dehydrated through ethanol series (70%, 85% and 95%) and air-dried. Fifteen micro-liters of hybridization mixture [50% (v/v) deionized formamide, 10% (w/v)

dextran sulfate, sonicated salmon sperm DNA (100 ng/ml), and 2-5 ng biotinylated probe DNA and 2x SCC], were added to each slide and then covered with a coverslip and sealed with rubber cement. After denaturation for 2 min on a hot plate at 80°C, the slides were incubated at 37 C for in a humid condition for at least 15 h. After hybridization, the coverslips were removed by dipping the slides in 2x SSC. The slides were then transferred in 50 % (v/v) formamide for 15 min at 37°C, washed twice in 2x SSC for 10 min each, and rinsed with 4x SSC for 5 min. For signal detection, the slides were treated with blocking agent [4% (w/v) Block Ace (Dainippon Pharmaceuticals) in 4x SSC] for 15 min at 37°C, followed by staining with 5 µg/ml avidin-fluorescein isothiocyanate (FITC) (Boehringer) in 4x SSC containing 1% (w/v) Block Ace for 45 min at 37 C. The slides were then washed at room temperature in 4x SSC, 4x SSC with 0.1% (w/v) Triton-X, and 4x SSC for 10 min each, and rinsed with 2x SSC for 5 min. Finally, the slides were mounted with anti-fade solution containing DAPI (1 µg/ml)/PI (0.5 µg/ml) for counter staining. Observation was made with a Nikon E600 epifluorescence microscope equipped with UA-1A and B-2A cubes. Micrographs were taken with an Olympus DP-70 CCD camera attached to the microscope.

RESULTS

Cytological features of mini-chromosome

Chromosomes of both strains prepared using GTBM without TBZ treatment were less condensed with the observable extra morphological details compared to the previous preparations in Chapter IV. DAPI-stained chromosomes appeared relatively longer and thread-like with highly stained intercalary and terminal knobs in both early metaphase and metaphase (Fig. V. 1A-B). With DAPI/PI staining, intensely fluorescing segments (IFSs) were much clearer, reflecting its highly heterochromatic nature (Fig. V. 1C). Unexpectedly, the mini-chromosome in strain ATCC18098 was dot to elliptical shape of almost the same size (ca. 0.5

μm in length) as the previous preparations with no distinct features. This mini-chromosome was totally stained with DAPI other than PI (Fig. V. 1C), suggesting highly AT content. Chromosome of similar size was never found in strain ATCC18099.

The dropping method that was newly applied in this study was shown to have a merit in obtaining chromosome specimens at relatively high frequency. However, most specimens prepared by this method were in prophase to early metaphase (Fig. V. 2A-C), with stretched or elongated chromosomes. Thus, morphological features were difficult to discern for these chromosomes. Mini-chromosome was slender, rod-like shape with the terminal knob on each end and was ca. 1.2 μm in length. In the metaphase, however, its size was reduced to 0.8 μm in length and terminal knobs were more distinctly stained with DAPI, thus suggesting they are AT-rich (Fig. V. 2C).

Florescence *in situ* hybridization

The dropping method has an advantage in visualizing the mini-chromosome, but the background cytoplasm and sometimes cell debris made specimens unsuitable to FISH analysis. So, chromosome painting FISH was carried out on the specimens of the two strains prepared by GTBM. Using the 410-kb chDNA isolated from PFGE as a probe (Fig. V. 3), probe hybridized to a contracted single spot in the nucleus of ATCC18098, proving that the 410-kb band is linked to DNA in the nucleus not in the cytoplasm. DAPI staining of the same nuclei showed that the hybridization spot corresponded to a minute AT- rich region in some nuclei and did not correspond at all in other nuclei. With mitotic metaphase specimens, the probe hybridized to the mini-chromosome and hybridization signals were not detected elsewhere. In strain ATCC18099, no hybridization signals were detected either with nuclei or with mitotic specimens.

These results confirmed that the 410-kb band on the PFGE gel represents the mini-chromosome detected in cytological observation and that it is not a derivative of A chromosomes of either strains. The dot-like appearance of signals in the interphase nucleus indicates that the mini-chromosome is not decondensed, but more or less condensed even in interphase. Interestingly, there was a difference in the AT-rich region pattern within the interphase nuclei of both strains, nuclei of ATCC18098 had almost the double number of AT-rich regions to those of ATCC19099.

DISCUSSION

This study showed that strain ATCC18098 contains a mini-chromosome that corresponds to the 410-kb chDNA detected by PFGE and cytology. The smallest records of fungal chromosomes visualized under a light microscope are 245-kb meiotic chromosomes of *S. cerevisiae* (Kuroiwa *et al.* 1984) and 350-kb mitotic chromosome of *Mycosphaerella graminicola* (Mehrabi *et al.* 2007). Therefore, this 410-kb mini-chromosome is the third smallest ever seen by light microscopy. Compared to the mini-chromosomes observed with GTBM (Taga *et al.* 1999, Chuma *et al.* 2003, Mehrabi *et al.* 2007, Garmaroodi and Taga 2007), morphological features of this chromosome could be observed in more details with the specimens prepared by the dropping method and this method seems to be one of the choices for preparation of mitotic specimens of other fungi. However, chromosomes spreads obtained by this method were mostly in prophase or early metaphase which may be related to fixation conditions. Applying of ice cold treatment may be helpful for arresting nuclei starting their division or during late prophase. Keeping in mind the extremely short time of mitosis in fungi, for example, mitotic time course of *Fusarium oxysporum* was early prophase 20 s, late prophase 50 s, metaphase 120 s, anaphase 13 s, early telophase 65 s and late telophase 60 s (Aist and Williams 1972), other explanations may be true.

In animals and plants, various chromosome painting techniques of FISH were proven to be useful for analyzing B chromosome (Rezacova *et al.* 2003, Muller *et al.* 2003, Liehr *et al.* 2004, Houben *et al.* 2001, Lysak *et al.* 2001, Schubert *et al.* 2001, Shishido *et al.* 2001, Nagy *et al.* 2002). In filamentous fungi, Taga *et al.* (1999) introduced similar FISH techniques for the identification of the conditionally dispensable (CD) chromosome in *N. haematoccca* MPVI. However, in this study, FISH was used to confirm that that the 410-kb chDNA band of PFGE corresponds to the mini-chromosome that was cytological visualized in the strain ATCC18098. Minute chromosomes like this 410-kb mini-chromosome that is present in certain strains but absent from others are likely to be dispensable chromosomes (Covert 1998). Absence of its sequence from strain ATCC18099 had proven its dispensability. Combining this with the facts that both strains have karyotypes with similar chromosome complements except this 410-kb mini-chromosome, it is reasonable to conclude that the basic CN for this fungus is n=9.

Unlike other mini-chromosomes that are mostly of heterochromatic nature and usually share sequences with A-chromosomes, those characteristics were not confirmed for the 410-kb chromosome. It was totally stained with DAPI in GTBM preparations but FISH signals on interphase nuclei were not correlated to heterochromatic region. Moreover, only the two terminal knobs of the mini-chromosome were relatively AT-rich in dropping method specimens, suggesting its euchromatic nature. Also, FISH results showed that this chromosome is not a derivative of any other chromosome in the genome. At least in this respect, it resembles to the CD chromosomes of *N. haematococca* MPVI (Taga *et al.* 1999, Garmaroodi and Taga 2007) and *Alternaria alternate* (Akagi per. comm.) in which chromosome painting FISH with the DNA from CD chromosome yielded the same results as those obtained here. Further analysis comparing to the other strains are needed to confirm the biological significance of this min-chromosome

and whether it is related to the virulence of strain ATCC18098 (Hawthorne *et al.* 1994) as with the other CD chromosomes.

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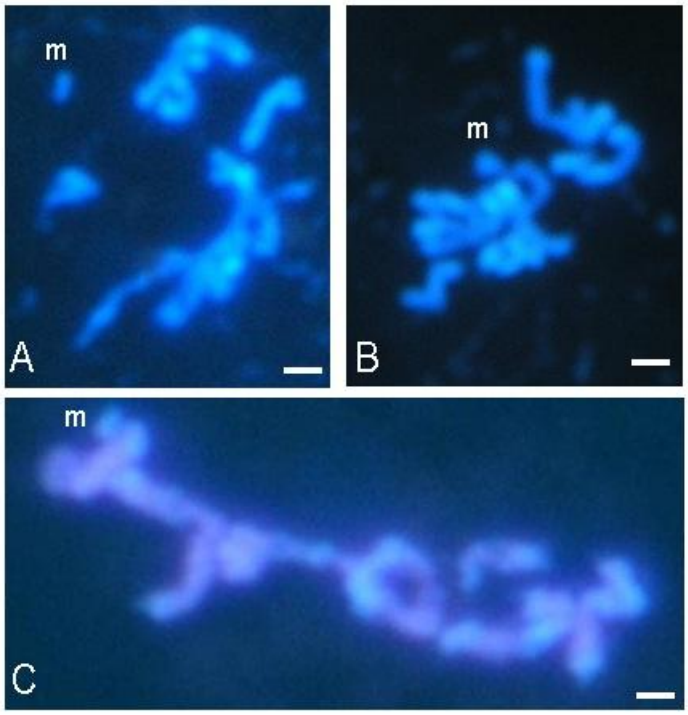

Fig. V. 1. Mini-chromosome visualization using GTBM without TBZ treatments, A- B. chromosome condensation at late prophase and early metaphase stained with DAPI. Mini-chromosome (m) is almost 0.5μm. C. metaphase chromosomes stained by DAPI/PI. AT-rich regions were evident, the mini-chromosome (m) was almost all of heterochromatic nature.

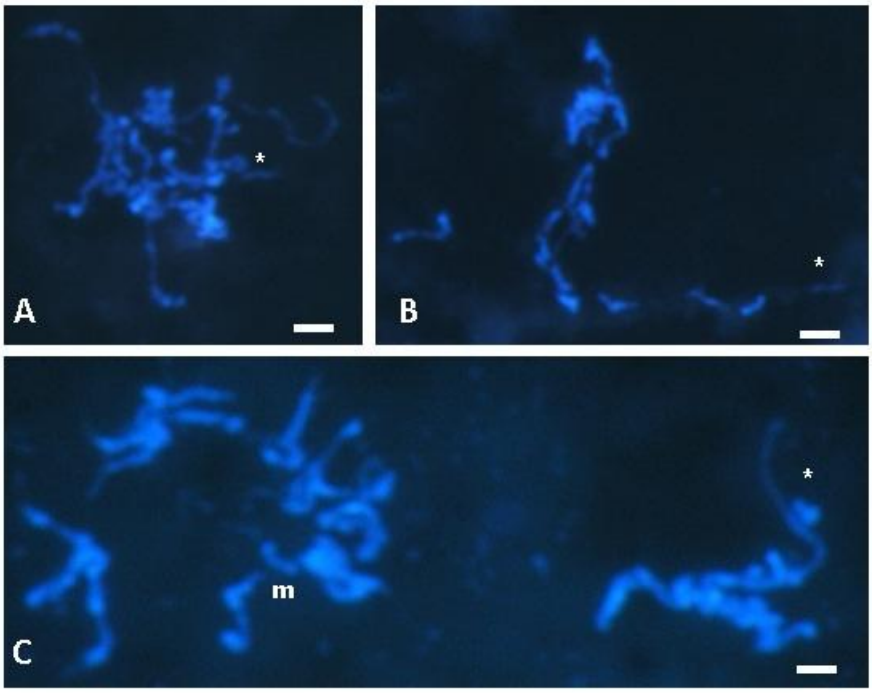

Fig. V. 2. Chromosome spreads at mitosis using dropping method and stained with DAPI. A-B. Chromosomes at prophase. AT-rich chromatin was evident in every chromosome. C. Chromosomes spread during early and mid-metaphase. Mini-chromosome was almost 1.0 -1.2 μm in size. Stars (*) indicates the suggested smallest chromosome in the spread which corresponds to the mini-chromosome and (m) refers to the mini-chromosome in concern.

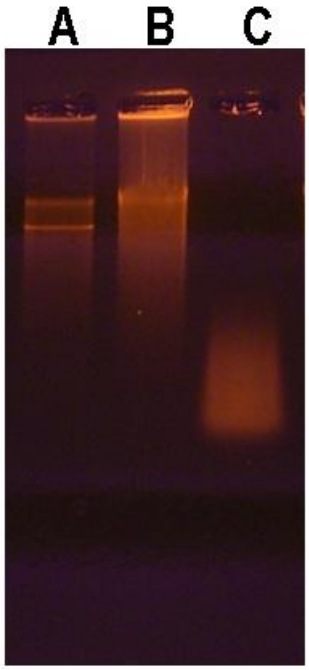

Fig. V. 3. Probe preparation for FISH from 410-kb chromosomal DNA (chDNA). A. DNA extracted from PFGE band using GeneClean II kit. B. DNA amplification using REPLI-g kit. C. Biotinylated DNA labeled with biotin-14-dATP by nick translation using a BioNick Labeling System. One μl of each sample was used for running on 1.2% agarose gel, 100 V, 30 min.

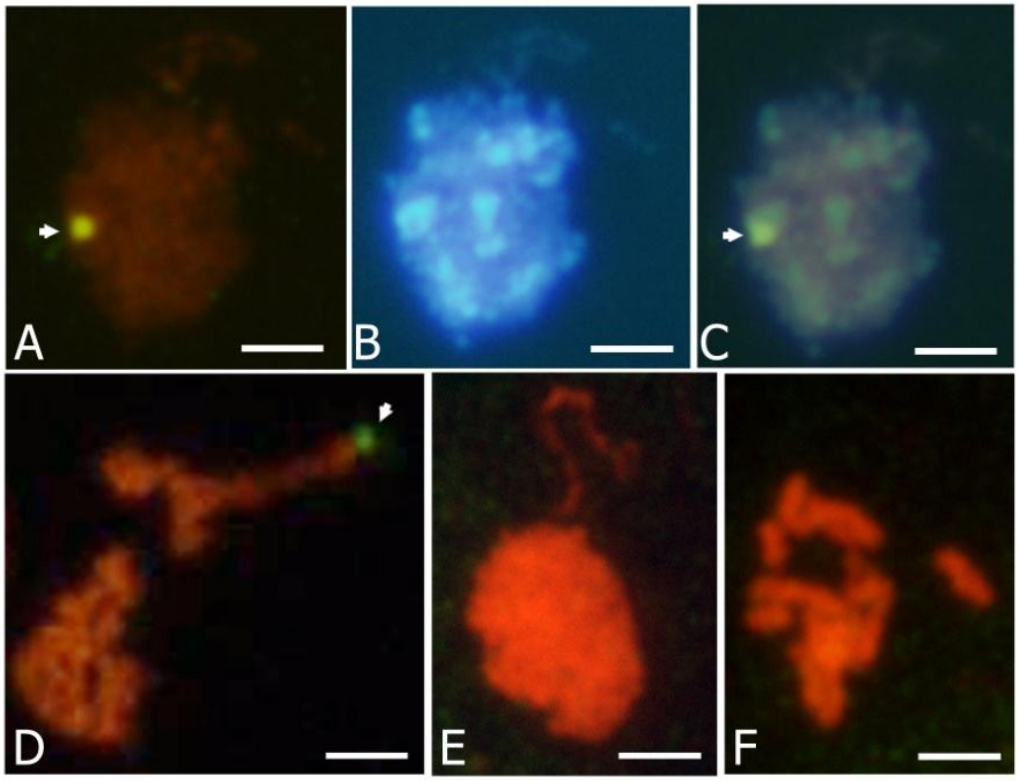

Fig. V. 4. Fluorescence *in situ* hybridization of the mini-chromosome in the genome of strain ATCC18098 using the 410-kb chDNA from PFGE as a probe. A. Hybridization signal (arrow head) of one interphase nucleus of strain ATCC18098. B. DAPI-stained image of the same nucleus as in A. C. Digitally overlaying both A and B. The signal partially overlaid a DAPI highly stained region. D. Hybridization signals on the spread of metaphase chromosomes of same strain. The whole mini-chromosome was painted by the probe (green), whereas the other chromosomes were not painted (red). E - F. Hybridization result of one interphase nucleus (E) and on the spread of metaphase chromosomes (F) of strain ATCC18099. No signals were detected. Bars: 2 μm.

CHAPTER VI

INHERITANCE MODE OF 410-KB MINI-CHROMOSOME IN *NECTRIA HAEMATOCOCCA* MPI

SUMMARY

Inheritance mode of the 410-kb chromosome was studied by tetrad and random ascospore analysis with ascospores obtained from reciprocal crossings between ATCC18098 and ATCC18099. Besides the mini-chromosome, segregation of MAT and perithecial color was examined in both types of ascospore analyses. The results of tetrad analysis indicated 4:4 segregation for the occurrence and absence of mini-chromosome in each tetrad, suggesting a Mendelian mode of inheritance. In random ascospore analysis using 90 ascospore progenies, chi-square test supported that progeny with and without mini-chromosome segregated in 1:1 in each cross. With 40 ascospores selected randomly from each cross, linkage analysis showed that the mini-chromosome segregated independently from either MAT or perithecial color trait, which indicates random assortment of the mini-chromosome and the other two chromosomes.

INTRODUCTION

Supernumerary or dispensable chromosome in fungi is defined as extra chromosome that is not present in at least another normally growing isolate of a species. Such chromosomes have so far been discovered in many fungal strains mainly by PFGE. While some of these chromosomes contain genes involved in virulence or pathogenicity and are named conditionally dispensable, others don't (for review see Covert 1998). During meiosis, some supernumerary chromosomes were inherited lesser than been expected by Mendelian genetics in some fungi (Miao *et al.* 1991, Orbach *et al.* 1996, Xu and Leslie 1996), whereas, the supernumerary chromosome of *Cochliobolus heterostrophus* was inherited by

more progeny than predicted (Tzeng *et al.* 1992). Also, these chromosomes may undergo structural rearrangement during meiosis (Francis and Michelmore 1993, Leclair *et al.* 1996, Miao *et al.* 1991, Tzeng *et al.* 1992, Xu and Leslie 1996, Wittenberg 2009) and also during post-meiotic mitosis (Chuma *et al.* 2003), which leads to chromosome polymorphisms within the progenies that are usually detected by PFGE and southern hybridization using one or several chromosome specific sequences.

While some fungi cannot be subjected to crossing experiments, *N. haematococca* MPI is well suited to genetic analyses because it is a heterothallic and its sexual stage can be obtained easily in laboratory conditions. Both tetrad and random ascospore analysis are possible and many traits relating to sexual reproduction, cultural appearance, heterothallism and tolerance to fungistatic compounds have been studied for this fungus through meiosis (for reviews, see Snyder *et al.* 1975, VanEtten and Kistler 1988).

In this chapter, the 410-kb supernumerary chromosome discovered in the strain ATCC18098 during the course of karyotyping was genetically analyzed for the meiotic inheritance mode.

MATERIALS AND METHODS

Fungal strains and culture

Two hermaphrodite strains of *N. haematococca* MPI were used in this study, ATCC18098 is *MAT1-1* and produces red perithecia as a female and ATCC18099 is MAT1-2 with white perithecia as a female. PDA and PDB were used for routine culture and for producing hyphae and budding cells, respectively. V8-juice agar (M-29, Stevens 1974) was used for crossing experiments.

Reciprocal crossing

Reciprocal crossing between ATCC18098 and ATCC18099 were made by the modified procedure of VanEtten (1978). Five ml of conidial suspension obtained by sweeping the surface of PDA culture plates of each strain were separately spread on V8 juice agar in plastic Petri dishes (10-cm diameter) and were allowed to develop by incubating at 22–24°C for 10–15 days under continuous fluorescence lighting. For spermatization, about 10 ml of conidial suspension prepared from the V8 juice plate culture was poured on the mycelia of the strain acting as the female. After 5 min, excess conidial suspension was drained, and the fertilized cultures were incubated as above for 2–3 weeks to allow formation of mature perithecia.

Ascospores analysis

For random ascospore analysis, the ascospore masses oozing from mature perithecia were suspended in distilled water and spread on 4% (w/v) water gelatin plates. Single ascospores were randomly isolated using a hand-made flexible glass needle and cultured on PDA slants. In tetrad analysis, apparently mature perithecia from each cross were picked up and washed with water to remove any conidia on their surface, and then squashed with a pair of forceps in a drop of water on the surface of a clean slide glass under a stereomicroscope. Rosette of asci were picked up by hand-made flexible glass needle and transferred to 4% gelatin plates. Individual ascus was moved to a new gelatin plate and ascospores were isolated from each ascus using the needle as before. Similarly, isolated ascospores were transferred to PDA slants. After PDA slants were incubated at 22- 24°C for 2-3 days, germinated ascospore progenies were selected, classified and used for inoculating SNA slants for long preservation cultures and on PDA plates for routine use under the same cultural conditions. SNA slants were moved to 4°C incubator 2-3 day after.

Pulsed field gel electrophoresis

Micorconidia-agarose plugs that contained microconidia in agarose were prepared by the modified method of McCluskey *et al.* (1990) as follows. After ascospore progenies were allowed to grow on PDA plates, small mycelial agar blocks cut from the culture were inoculated to 40 ml of PDB medium in a 100 ml flask and incubated on a rotary shaker at 220 rpm for 4-6 days. The resulting culture was filtered through one layer of Kimwipe and washed twice by centrifugation in distilled water. TSE buffer (25 mM Tris-HCl, pH 7.5, 1 M sorbitol and 25 mM EDTA, pH 8.0) was added to the final pellets to make conidial suspensions of 0.8–1 × 10^8/ml. An equal volume of 2.5% (w/v) low-melting agarose in TSE was added, mixed, and put in the mold of PFGE plug. The solidified plugs were transferred to lysis buffer [1% (w/v) SDS, 1 mg/ml proteinase K and 0.5 M EDTA, pH 8.0] and incubated at 50°C for 24 h with gentle shaking. The plugs were then rinsed with 0.5 M EDTA three times for 30 min each and finally stored in fresh 0.5 M EDTA solution at 4°C until use. Detection of the mini-chromosome by PFGE was carried out with CHEF DRII and was resolved on 0.8% (w/v) agarose gels using 0.5× Tris-borate-EDTA running buffer. Running conditions were: 200 V with ramping pulse of 120 s for 15 h followed by 180 s for 8 h. For size marker, chDNAs of *S. cerevisiae* was used. Gel staining and visualization was performed as in Chapter II.

Determination of mating type (MAT) and the perithecial color traits

In both types of ascospore analyses, MAT and the perithecial color were detected to check if segregation in sexual crosses was normal for the other chromosomes. For the unordered tetrad analysis, both traits were determined by back-crossing of each ascospore progenies with each parental strain. In random ascospores

analysis, MAT was detected with PCR then back-crossed with the parental strain of opposite MAT parental strain to detect the perithecial color.

Determination of MAT by PCR

MAT gene for 40 random ascospore progenies were detected using PCR amplification. Total DNA as template for PCR was isolated from 3-days-old mycelia grown in PDB by using the procedure described by Garmaroodi and Taga (2007). For the PCR determination of MAT, a new primer set was designed for *MAT1-1* (Nh98-MAT1-F2, CGCCCTCTGAATGCCTTTAT, Nh98-MAT1-R2, CGCATGATAGGGCAGCAA) based on the sequence information of other *Fusarium spp*. (AJ535625, AF318048, 77-13-7, AY040737, AJ535626, AJ535627, and AJ535628). These primers were tested with ATCC18098 and ATCC18099 as well as many progeny with known *MAT* detected earlier. *MAT1-2* was amplified with the degenerate primers fusHMG-for (CGACCTCCCAAYGCYTACAT) and fusHMG-rev (TGGGCGGTACTGGTARTCRGG) as described by Kerenyi *et al.* (2004). PCR amplification was done in 10 µl PCR reaction mixtures containing 1 ng/µl of template DNA, 2 µM each of primers, 5 µl GoTaq Green master mix (Promega). PCR amplification conditions were: initial denaturation at 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. Amplification of the expected fragment was analyzed by gel electrophoresis through a 1.2% agarose gels in 1× Tris-acetate EDTA buffer.

Randomly amplified polymorphic DNA analysis

The 410-kb chDNA of ATCC18098 to be used as a template was extracted from the gel of the second run using GeneClean II kit (BIO 101) as described in Chapter V. PCR amplification was done in 10 µl PCR reaction mixtures containing 1 ng/µl of template DNA, 1 µM each of primers and 5 µl GoTaq Green

master mix (Promega). Amplification conditions were: initial denaturation at 94°C for 1 min, followed by 40 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min. Amplified fragments were analyzed by gel electrophoresis with a 1.2% agarose gels in 1× Tris-acetate EDTA buffer as a running buffer. A total of 100 decamer primers purchased from Operon Technologies Inc. (USA), that is, OPA1-20, OPE1-20, OPG1-20, OPQ1-20 and OPS1-20, were tested for the amplification of RAPD bands. Reproducibly amplified fragments were scored and compared with the fragments amplified by the same primer using the total DNA from each parental strain as a template. The bands unique to ATCC 18098 were selected and DNA was extracted from that bands using QIAquick gel extraction kit (Qiagen), cloned using TOPO TA cloning kit (Invitrogen) and sequenced using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and ABI PRISM 310 Genetic Analyzer.

RESULTS

Inheritance of mini-chromosome through sexual crossing

The inheritance mode of the mini-chromosome in sexual crosses was studied by tetrad and random ascospore analysis with ascospores obtained from reciprocal crossings between ATCC18098 and ATCC18099 (Fig. VI. 1). For detecting mini-chromosomes in the progenies by PFGE, microconidia-agarose plugs instead of protoplast-agarose plugs were used because of the relatively simple procedure of plug preparation. With these plugs, the bands representing mini-chromosome were separated at the expected position throughout the study, which suggested that the mini-chromosome did not undergo chromosome rearrangements during meiosis (Fig. VI. 2).

As an alternative to PFGE for detection of this mini-chromosome, construction of a PCR primer set that specifically amplify mini-chromosome was attempted using RAPD primers. Single primers were tested using DNA of the

410-kb chromosome as a template. Out of 100 primers tested, ten primers were positive and the number of amplified fragments ranged from 1-4 (Table VI. 1, Fig. VI. 3a). Comparison of the banding pattern for each primer showed that Op-Q01 amplified two bands that were specific to both the mini-chromosome and ATCC18098 (Fig. VI. 3b). Using Op-Q01 with genomic DNAs of six ascospore progenies from an unordered tetrad showed that one band of ca. 450bp in size was more intensely amplified in the ascospore progenies harboring the mini-chromosome (Fig VI. 3c). Thus, primer Op-Q01 with the 450 bp fragment was used for pre-detection of the mini-chromosome in random ascospores progeny. This 450bp band was cloned and sequenced (GeneBank access no. HQ013300). Blast search for the sequence of this band showed 80% resemblance with one clone fragment associated with chromosome 8 in *N. haematococca* MPIV.

Unordered tetrad analysis

In tetrad analysis, many sets of eight ascospores were isolated from asci, but no complete tetrads (or more precisely, octads) were obtained, perhaps due to failure of ascospore germination. Consequently, a total of 11 incomplete tetrads with six or seven germinated ascospores were subjected to analyses by deducing the phenotypes of ungerminated ascospores. The reconstruction of full tetrad was based on the assumption that each of the four meiotic products was duplicated by post-meiotic mitosis to yield eight ascospores in an ascus (Table VI. 2). Irrespective of whether the mini-chromosome was transmitted from female or male, each of the reconstructed tetrads showed 4:4 segregation for the occurrence and absence of mini-chromosome, suggesting that this chromosome was inherited in a Mendelian manner (Table VI. 3).

Random ascospores analysis

In random ascospore analysis, a total of 90 ascospores were analyzed for the segregation of mini-chromosome in each of the reciprocal crossings (Table VI. 4).

Chi-square test supported that progeny with and without mini-chromosome segregated in 1:1 in each cross, again suggesting Mendelian inheritance of this chromosome. Subsequently, random assortment between the mini-chromosome and other chromosomes during meiosis were examined using MAT and perithecial color as the markers. With 40 ascospores selected randomly from each cross, linkage analysis showed that the mini-chromosome segregated independently from either trait. This indicates random assortment of the mini-chromosome and the other two chromosomes. It is probable that the same relationship exists between the mini-chromosome and the rest of the chromosomes in the genome. Also, both MAT and perithecial color were shown to be controlled by single genes and independently inherited (Table VI. 4).

DISCUSSION

To study the inheritance mode of the 410- kb mini-chromosome discovered in strain ATCC18098, the ascospore progenies of the crossing between this strain and strain ATCC18099 were subjected to molecular analysis for the presence of the mini-chromosome. Although PCR detection may be usable, PFGE was used in detection of mini-chromosome for all the progenies of unordered ascospores as well as the random ascospore progenies used in data analysis. Structural changes due to deletion, inversion, translocation and duplication were reported for other mini-chromosomes (Han *et al.* 2001, Hatta *et al.* 2002, Wang *et al.* 2003), in which chromosomes length polymorphism within the meiotic progenies was observed (for examples, Maio *et al.* 1991, Tzeng *et al.* 1992, Francis and Michelmore 1993, Leclair *et al.* 1996, Xu and Lieslie 1996, Enkerli *et al.* 2000, Chuma *et al.* 2003). Such changes cannot be traced by PCR-based analysis. In this study, PFGE confirmed that the 410-kb mini-chromosome did not undergo structural changes in size or number during meiosis which is peculiar compared to mini-chromosomes in some other fungi. Microconidia-agarose plugs used here are less coasty and more speedy to prepare than the method using protoplast-agarose plugs.

Therefor the method and the protocol described here are thought to be suitable for the separation of other mini-chromosomes as well.

PCR was efficiently used for MAT detection using the universal primer set (Kerenyi *et al.* 2004). In this study, the one primer set designed based on the fact that MAT is conservative within the *Fusarium* species worked well. Similarly, I tried to degenerate PCR specific marker for this mini-chromosome through the utility of RAPD primes (Williams *et al.* 1990), although PFGE was preferred in detecting the mini-chromosome within the genome of the ascospores progenies. RAPD primers were used to assess genome variability between 21 isolates from MPI and MPV (Crowhurst *et al.* 1991). They could distinct the two mating groups and generated race specific hybridization probes that were used later in phylogenetic analysis (O'Donnell 2000). Such similarity could be the reason why only one specific amplified fragment could be detected in this study. Unfortunately, this fragment was too short to be rely on.

Tetrad analysis in this study suggested that the 410-kb mini-chromosome was transmitted in a Mendelian manner in crosses between ATCC18098 and ATCC18099. Two explanations are possible for this. One is that the two sister chromatids moved to one pole in meiosis I, followed by the separation of sister chromatids in meiosis II to yield a 2:2 ratio for the occurrence and absence of the chromosome in a tetrad. The other is that sister chromatids separated at meiosis I instead of meiosis II in a manner called pre-division or premature centromere division (Fig. VI. 4). Examples of such division have been reported in fungi (Fulton and Bond 1983) and animals (Angell 1991). Cytologically distinguishing between the two hypotheses was failed due to the difficulty to discern the mini-chromosome in the cluster of meiotic chromosomes and the inability to perform FISH analysis inside asci.

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Table VI. 1. Random amplified polymorphic DNA (RAPD) primers sequences amplified fragments of the 410-kb mini-chromosome.

RAPD Primer	Sequence (5'-3')	Number of bands
OPA-02	TGCCGAGCTG	2
OPA-03	AGTCAGCCAC	1
OPA-04	AATCGGGCTG	1
OPA-05	AGGGGTCTTG	1
OPA-08	GTGACGTAGG	2
OPE-07	AGATGCAGCC	3
OPE-14	CTACTGCCGT	4
OPQ-01	GGGACGATGG	2
OPS -03	CAGAGGTCCC	2
OPS-07	TCCGATGCTG	1

Table VI. 2. Unordered tetrad results for the segregation of the 410-kb mini-chromosome, MAT and perithecial color gene.

Ascus	Ascospore	Mini-chromosome.	MAT1-	Perithecial color
A	1	-	1	W
	2	-	1	W
	3	-	2	R
	(m)	(-)	(2)	(R)
	5	+	1	R
	(m)	(+)	(1)	(R)
	4	+	2	W
	6	+	2	W
B	1	+	1	R
	3	+	1	R
	5	+	2	R
	(m)	(+)	(2)	(R)
	2	-	2	W
	6	-	2	W
	4	-	1	W
	(m)	(-)	(1)	(W)
C	1	+	1	W
	4	+	1	W
	5	+	2	R
	(m)	(+)	(2)	(R)
	2	-	2	W
	3	-	2	W
	6	-	1	R
	(m)	(-)	(1)	(R)
D	4	+	1	W
	6	+	1	W
	2	+	2	R
	5	+	2	R
	3	-	1	R
	(m)	(-)	(1)	(R)
	1	-	2	W
	(m)	(-)	(2)	(W)

Ascus	Ascospore	Mini-chromosome.	MAT1-	Perithecial color
E	1	+	2	R
	(m)	(+)	(2)	(R)
	4	+	2	W
	6	+	2	W
	2	-	1	R
	3	-	1	R
	5	-	1	W
	(m)	(-)	(1)	(W)
F	2	+	1	W
	4	+	1	W
	3	+	1	R
	(m)	(+)	(1)	(R)
	5	-	2	W
	6	-	2	W
	1	-	2	R
	(m)	(-)	(2)	(R)
G	1	-	1	W
	2	-	1	W
	5	-	1	W
	(m)	(-)	(1)	(W)
	3	+	2	R
	4	+	2	R
	6	+	2	R
	(m)	(+)	(2)	(R)
H	1	+	1	R
	3	+	1	R
	5	+	2	R
	(m)	(+)	(2)	(R)
	2	-	1	W
	4	-	1	W
	6	-	2	W
	7	-	2	W
I	2	+	2	W
	4	+	2	W
	3	+	2	R
	(m)	(+)	(2)	(R)
	1	-	1	R
	6	-	1	R
	5	-	1	W
	(m)	(-)	(1)	(W)

Ascus	Ascospore	Mini-chromosome	MAT1-	Perithecial color
J	1	+	2	W
	4	+	2	W
	5	+	2	R
	(m)	(+)	(2)	(R)
	2	-	1	W
	3	-	1	W
	6	-	1	R
	(m)	(-)	(1)	(R)
K	5	+	1	W
	6	+	1	W
	1	+	2	R
	(m)	(+)	(2)	(R)
	2	-	2	R
	3	-	2	R
	4	-	1	W
	(m)	(-)	(1)	(W)

(m): missing ascospore progeny; +, - : presence and absence of mini-chromosome; 1, 2: MAT1 genes; R, W: perithecial color.

Table VI. 3. Unordered tetrad analysis for the segregation of the 410-kb mini-chromosome.

Observed ratio	Crossing (female×male) : no. of asci analyzed	Speculated ratio[a]
3+: 4-	ATCC18099 × ATCC18098 : 1	4+:4-
4+: 2-	ATCC18098 × ATCC18099 : 1	4+:4-
3+:3-	ATCC18099 × ATCC18098 : 4	4+:4-
3+:3-	ATCC18098 × ATCC18099 : 5	4+:4-

[a]Speculated ratio in the reconstructed complete tetrad.

+ : 410-kb mini-chromosome detected; -: 410-kb mini-chromosome not detected.

Table VI. 4. Random ascospore analysis for the segregation of 410-kb mini-chromosome, MAT and perithecial color gene.

Cross (female × male)	Segregation		Total	Chi-square test	
	+[a]	-[a]			
ATCC18099 × ATCC18098					
MC	48	42	90	(1:1)	0.50<P<0.75
MC, MAT	*MAT1-1*:*MAT1-2*=12:13	*MAT1-1*:*MAT1-2*=8:7	40	(1:1:1:1)	0.25<P<0.50
MC, PC	red:white=13:12	red:white=5:10	40	(1:1:1:1)	0.25<P<0.50
MC, MAT, PC	*MAT1-1,* red:white=7:5 *MAT1-2,* red:white=6:7	*MAT1-1,* red:white=-3:5 *MAT1-2,* red:white=2:5	40[b]	(1:1:1:1:1:1:1:1)	0.50<P<0.75
ATCC18098 × ATCC18099					
MC	52	38	90	(1:1)	0.10<P<0.25
MC, MAT	*MAT1-1*:*MAT1-2*=9:10	*MAT1-1*:*MAT1-2*=9:12	40	(1:1:1:1)	0.75<P<0.90
MC, PC	red:white=10:9	red:white=8:13	40	(1:1:1:1)	0.50<P<0.75
MC, MAT, PC	*MAT1-1,* red:white=5:4 *MAT1-2,* red:white=5:5	*MAT1-1,* red:white=4:5 *MAT1-2,* red:white=4:8	40[c]	(1:1:1:1:1:1:1:1)	0.95<P<0.90

[a] +, mini-chromosome detected; -, mini-chromosome not detected; MC, mini-chromosome; PC, perithecial color.

[b] *MAT1-1*, red : *MAT1-1*, white : *MAT1-2*, red : *MAT1-2*, white =10 : 10 : 8 : 12. P of χ^2 test (1:1:1:1) is >0.9,

[c] *MAT1-1*, red : *MAT1-1*, white : *MAT1-2*, red : *MAT1-2*, white = 9 : 9 : 9 : 13. P of χ^2 test (1:1:1:1) is >0.9.

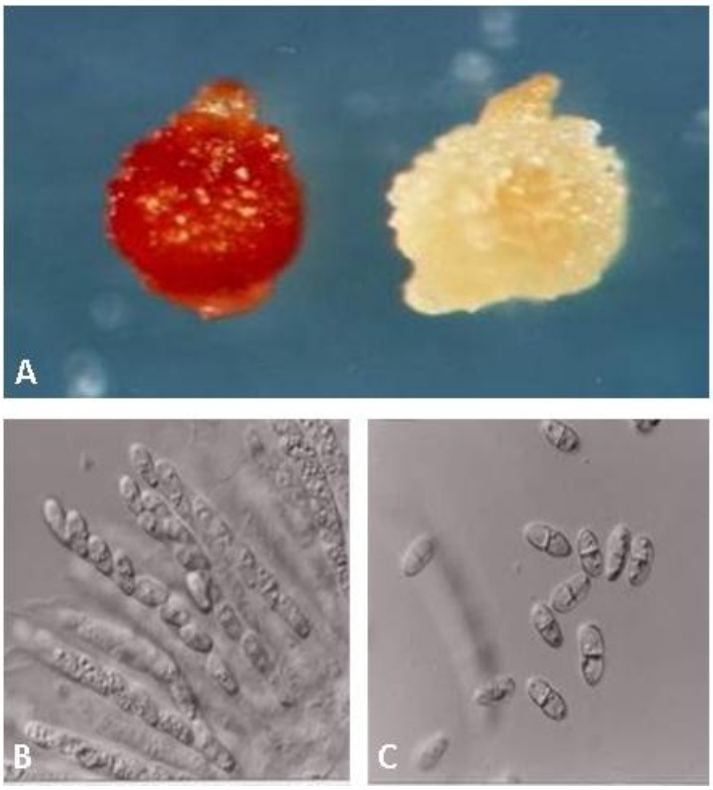

Fig. VI. 1. Reciprocal crossing of ATCC18098 X ATCC18099, A. Hermaphrodite perithecia resulting from the two reciprocal crossing distinguished by red color for female ATCC18098 and white for female ATCC18099. B, Ascospores within asci at different stages of division. Upon maturation, eight unordered ascospores per ascus arose. C. Mature ascospores released from asci. Each ascospore is two-celled.

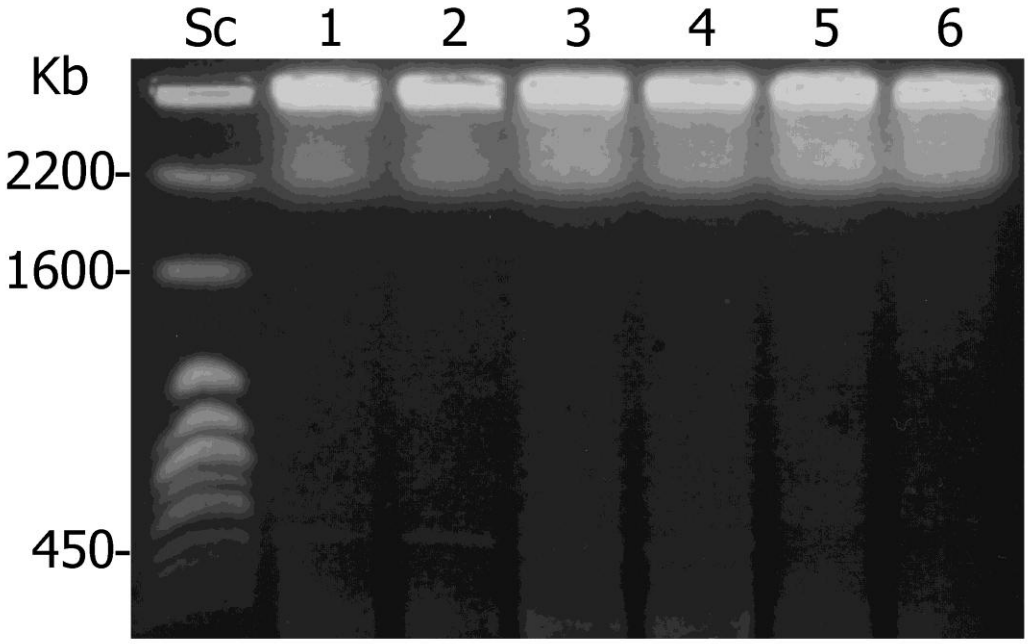

FIG. VI. 2. Detection of the mini-chromosome in ascospore progenies by pulsed field gel electrophoresis with microconidia-agarose plugs. Lanes 1 to 6 represent six ascospore progenies isolated from the single ascus. The bands representing the mini-chromosome are recognized in the lanes 1 to 4, but not in the lanes 5 and 6. Note that no large difference in band size is recognized among lanes 1 to 4. Running conditions were: 0.8% agarose, 180 V with constant pulse time of 120 s for 12 h and 180 s for 8 h. *Saccharomyces cerevisiae* (Sc) was used as DNA size standard. Numbers to the left of each panel indicate DNA sizes.

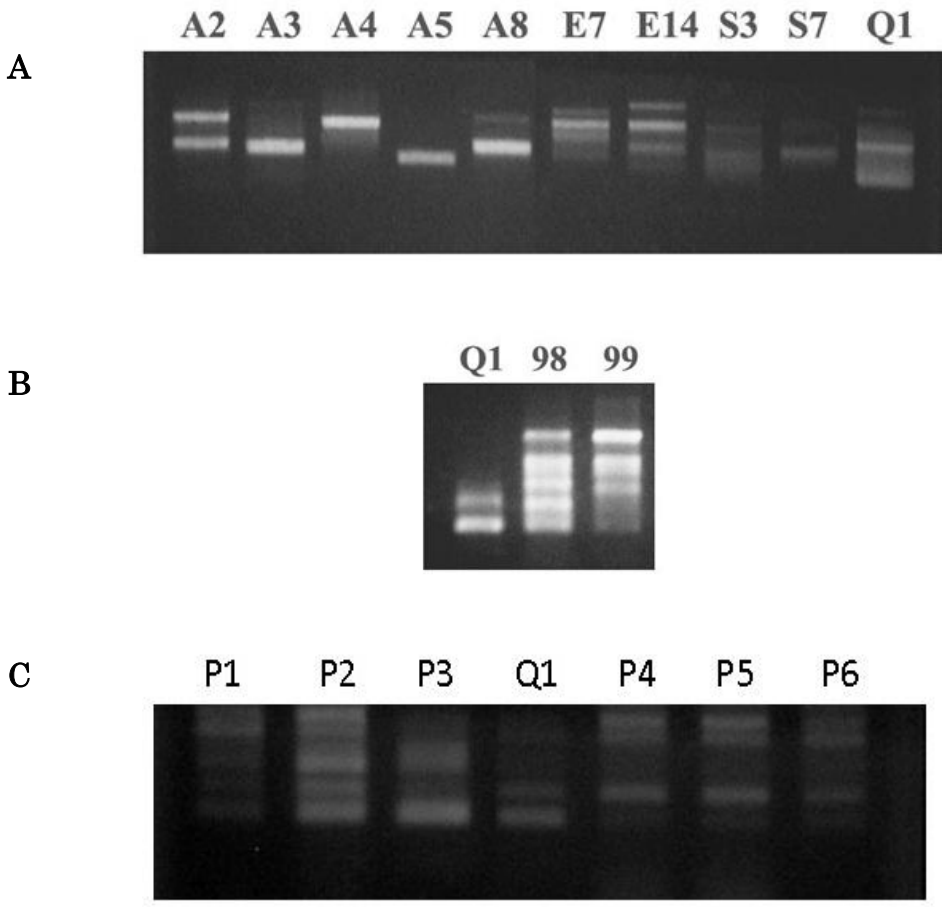

Fig. VI. 3. Amplification pattern with RAPD primers **A**. Different amplification fragments using oligo primers with the 410-kb chDNA from mini-chromosome as a template. **B**. Comparison between the banding pattern of OpQ-01 using the DNA from 410-kb chDNA and ATCC18098 and ATCC18099 genomic DNA as template, The two band are missing in the genome of ATCC18099. **C**. Banding pattern with the DNA extracted from six ascospores progenies resulting from one ascus, P1-P3, banding pattern for the ascospores containing mini-chromosome inherited, P4-P6 banding pattern for the ascospores without the mini-chromosome. Q1 the banding pattern using the chDNA from strain ATCC18098. The lower band is very faint in the progeny without the mini-chromosomes.

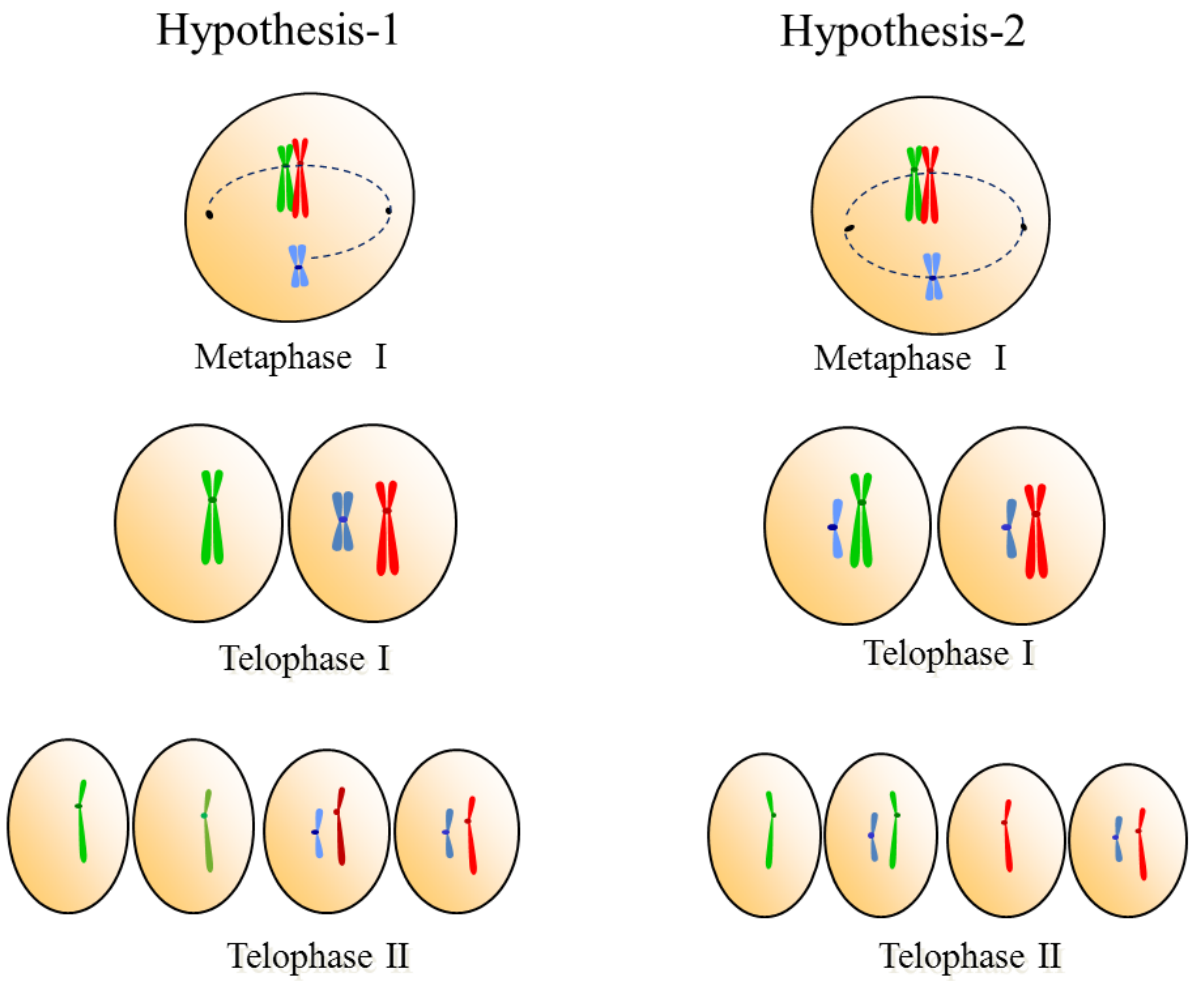

Fig. VI. 4. Interpretation of the 1:1 segregation for the 410-kb mini-chromosome. Hypothesis-1: centromere of mini-chromosome may behave normally during meiosis that it divides during metaphase II not during metaphase I. Hypothesis-2: premature division of centromere during metaphase I.

Appendix 1:

Nectria haematococca mpI RAPD marker NHmpI chro10 Q01-01 genomic sequence

GenBank: HQ013300.1

FASTA Graphics

Go to:

```
LOCUS       HQ013300               452 bp    DNA     linear   PLN 26-DEC-2010
DEFINITION  Nectria haematococca mpI RAPD marker NHmpI chro10 Q01-01 genomic
            sequence.
ACCESSION   HQ013300
VERSION     HQ013300.1  GI:315284493
KEYWORDS    .
SOURCE      Nectria haematococca mpI
  ORGANISM  Nectria haematococca mpI
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            Sordariomycetes; Hypocreomycetidae; Hypocreales; Nectriaceae;
            Nectria; Nectria haematococca complex.
REFERENCE   1  (bases 1 to 452)
  AUTHORS   Mahmoud Ahmed,A.M. and Taga,M.
  TITLE     Specific RAPD sequence from the 450-kb B chromosome in the
            filamentous ascomycetes Nectria haematococca mpI (anamoph, Fusarium
            solani f. sp. cucurbitae)
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 452)
  AUTHORS   Mahmoud Ahmed,A.M. and Taga,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-JUL-2010) Bioscience, Graduate School of Natural
            Science & Technology, Okayama University, Kitaku, Tsushima Naka
            3-1-1, Okayama, Okayama 700-0080, Japan
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      121 ctgaatacag tacgcacttt tgcacgacgc acttcgaacg cacgctgacg aacgatagac
      181 aacctgggtt tggttttgac atactgggaa actcctatcc cacgatgggg ctggatcatg
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      301 agctacacag gggaatcttc ctctcgttta ccttcctcgg tattcaaatc cttcggtgag
      361 gccgaattct ggcttgctct gatcaaggtc attgccatcg tcccaagccg aagatatcca
      421 agcacgactg gcggccgtta ctagatggaa tc
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CHAPTER VII

GENERAL CONCLUSIONS AND PERSPECTIVES

In this study, the karyotypes of two heterothallic strains of *N. haematococca* was investigated by using three different methods, namely conventional meiotic cytology, PFGE and mitotic cytology using GTBM. Prior to this study, the only available data for CNs of this fungus were obtained by meiotic cytology more than 50 years ago (Hirsh 1947, 1949, El-Ani 1954, 1956). Recently, the reliability of conventional meiotic cytology was questioned (Taga *et al.* 1998). As an important conclusion of this study is that conventional light microscopy with asci could lead to the underestimation of CN, thus confirming previous reports for other fungi (Taga *et al.* 1998, Tsuchiya and Taga 2001, Raju 2008). Since, this may be true for other filamentous fungi that have already been investigated using conventional method, reexamination is also necessary for such fungi.

Unfortunately, only a few reports of karyotype analysis that combined cytology and PFGE had been published in filamentous fungi (Orbach *et al.* 1988, 1996, Borbye *et al.* 1992, Taga *et al.* 1998, Akamatsu *et al.* 1999, Tsuchiya and Taga 2001, Gale *et al.* 2005, Mehrabi 2007, Eusebio-Cope *et al.* 2009). In many species their karyotypes cannot be determined solely by PFGE. For such species, reliable cytological method such as GTBM should be applied jointly. Similarly, reliable karyotyping for *N. haematococca* MPI has been achieved through the combination of the result obtained from both PFGE and GTBM using DAPI/PI staining in this study (Fig. VII. 1).

A 410-kb mini-chromosome was cytologically visualized in this study. GTBM can provide a reasonable estimate as to the CN and give information on chromosomal morphology, but detailed morphology of the 410-kb mini-chromosome was more clearly distinguished by the dropping method developed in this study. So far, cytological visualization of mini-chromosomes

was only done by GTBM (Taga *et al.* 1999, Chuma *et al.* 2003, Mehrabi *et al.* 2007). These mini-chromosomes appeared as a dot-like shaped in spite of that the sizes in the first two publications are 4 and 3 times larger than the 410-kb minichromosome. Considering the simplicity in preparing fungal chromosome spread using this method, I hope to break the apparent barriers to perform fungal cytology in most laboratories either in research or undergraduate levels. Combining this method with the DAPI staining of ascospore as introduced in Chapter III, both mitotic and meiotic investigation could be applied with simple procedures and reasonable resolution.

In this study, molecular techniques, such as PFGE, Southern hybridization, FISH, PCR amplification and RAPD were used for identification of the 410-kb supernumerary chromosome beside cytological visualization. Except for PFGE which was used extensively with fungi (Mills and McCluskey 1990, Zolan 1995, Covert 1998), other techniques were adopted from other eukaryotes and were successfully been applied to fungi. FISH, which had been proven to be useful for analyzing B chromosome in other eukaryotes (Rezacova *et al.* 2003, Muller *et al.* 2003, Liehr *et al.* 2004, Houben *et al.* 2001, Lysak *et al.* 2001, Schubert *et al.* 2001, Shishido *et al.* 2001, Nagy *et al.* 2002) was used to correlate molecular and cytological data and as a DNA/DNA hybridization tool (Taga and Murata, 1994) to confirm its supernumerary. PCR was used to identify MAT genes of ascospores progeny with both universal primers and a newly degenerate primer sets. RAPD primers gave useful information without the need to do whole chromosome sequencing. This chromosome with its size, morphology and Mendelian mode of inheritance is an amenable candidate for further molecular and genetic investigations to reveal its sequence, structure and biological significance, such investigations are currently among the main topics in fungal genetics and have been analyzed extensively to unravel the origin of both chromosome polymorphisms and supernumerary chromosomes.

Finally, the comprehensive karyotype obtained in this study for *N. haematococca* MPI not only participates in the study of the karyotype evolution in *N. haematococca-Fusarium* species complex which represents an ideal example of the evolution of biological species (Fig. VII. 2). That project t but also it may contribute to advancement of the genetics of this fungus. Several protocols had already been applied to elucidate this goal. DAPI staining of meiotic specimens, PFGE protocols, including the preparation of protoplasts and running conditions, cytological protocols with GTBM and dropping method, crossing. Such techniques may serve as a reference for further studies for electrophoretic and cytological karyotyping, mode of inheritance, gene mapping, and other genetic analyses. Also, this study addressed many questions concerning aggregation of chromosomes during meiosis, chromosome polymorphism and heterochromatin banding pattern of the two strains, the origin and biological importance of the 410-kb mini-chromosome

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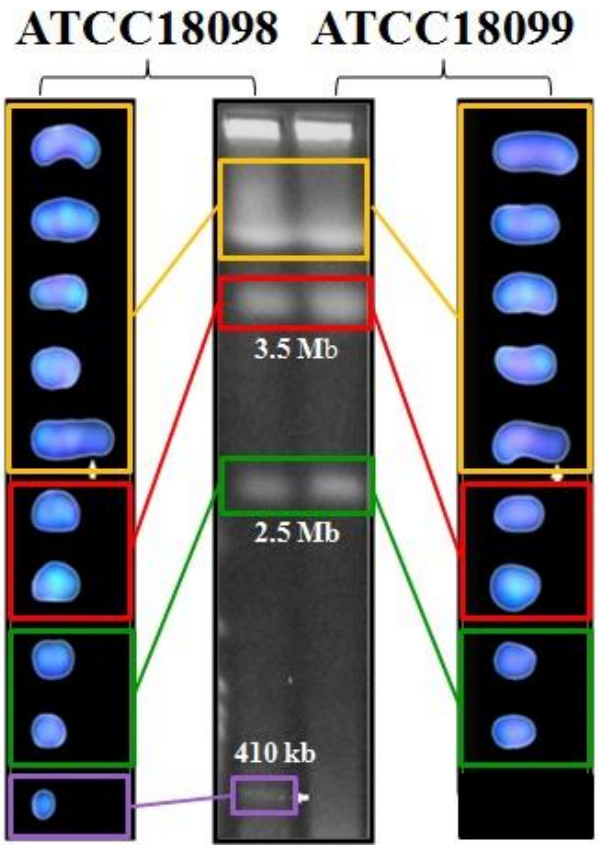

Fig. VII. 1. Integration of both Molecular and cytological karyotyping of the two strains of *N. haematococca* MPI. Five chromosomes are in the range of > 5.5 Mb. Both the 3.5 Mb and 2.5 Mb PFGE bands are thought to be doublet which are represented in chromosomes 6, 7 and chromosomes 8, 9 respectively. The 410-kb chDNA band is corresponding to the mini-chromosome in strain ATCC18098.

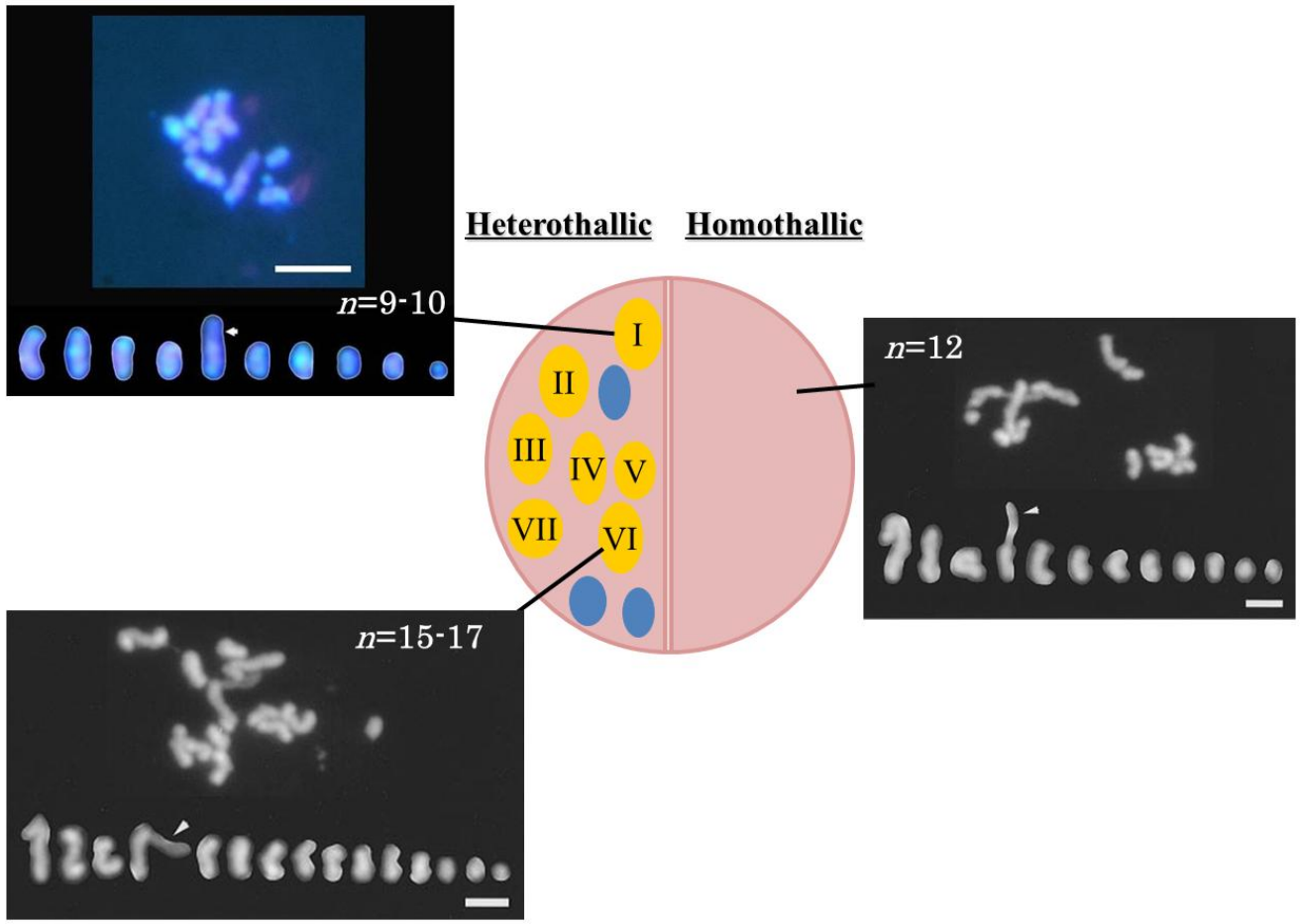

Figure VII. 2. Karyotype evolution in *N. haematococca* species complex through the comparison among the karyotype of different strains of *N. haematococca* obtained in this study and previous studies.