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Morphological Structure of Propagules and Electrophoretic Karyotype Analysis of False Smut *Villosiclava virens* in Rice

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The target pathogen *Villosiclava virens* (teleomorph: *claviceps oryzae-sativae*) was isolated from the infected rice, where it caused false smut. In our study, the forming processes of the chlamydospores, chlamydospore balls, conidiospores, and secondary conidiospores during the asexual reproduction were observed more precisely and in greater detail than previous descriptions. The microstructure of the infected rice kernel showed that the outer dense chlamydospores piled around the false smut balls grown on XBZ medium; moreover the sclerotia consisting of dense mycelium were found. The different morphology was observed across the different growing conditions. In addition, we observed the nuclear numbers of both the conidiospores and hyphae using 4′,6-diamidino-2-phenylindole (DAPI) staining. Because the fungus has small chromosomes and the numbers were not previously known, we analyzed the electrophoretic karyotype using a pulsed field gel electrophoresis (PFGE) technique. The results showed that *V. virens* has at least 10 chromosomes ranging in size from 0.6 kb to 6 Mb. The *V. virens* genome size is estimated to be 23 Mb. Here, we report the morphological characteristics of the fungus and the process of asexual spores forming asexual propagules, along with the first analyze the molecular karyotype of *V. virens*. These results supply a foundation for further study of the pathogenicity and biology of this devastating pathogen.

Keywords: false smut, propagulum, PFGE, *Ustilaginoidea virens*, XBZ medium

Introduction

Rice false smut is a worldwide fungal disease in rice (*Oryza Sativa* L.) and corn (*Zea mays* L.) caused by the *Villosiclava virens* (Nakata) E. Tanaka & C. Tanaka (Tanaka *et al*., 2008), teleomorph: *claviceps oryzae-ativae* Hashioka (Hashioka, 1971; Abbas *et al*., 2002), and anamorph: *Ustilaginoidea virens* (Cook) (Takahashi, 1896). Infection by the fungus transforms individual grains of infected panicles into greenish spore balls that have a surface which is covered by an abundance of powdery dark-green spores. False smut of rice has been largely ignored because of its only occasional occurrence in certain regions, but epidemics of the disease have recently been reported (Rush *et al*., 2000). The fungus can survive as sclerotia or hardened spore balls, that have been known to survive up to 4 mo under field conditions (Lee and Gunnell, 1992). In recent years, rice false smut has become a serious problem in part due to changes in climate and rice varieties. In 1988, rice production in the northern areas of Japan was severely damaged by infection with *V. virens* (Yaegashin *et al*., 1989). Previously known as a minor disease with occasional occurrence in China in the 1950s, rice false smut has become widespread to all of rice-growing areas by the wide adoption of high-yielding semi-dwarf rice cultivars and the heavy application of N fertilizer since the 1980s, and it has emerged as the most devastating grain disease of rice in the last 20 years in the major rice-growing regions of North, the Yunnan Province and along the Yangtze River in China (Deng, 1989). In addition, the fungus produces ustiloxin, a phytotoxin and mycotoxin, in the diseased plant tissues. The ustiloxin is an inhibitor of microtubules (Luduena *et al*., 1994) and makes the contaminated rice products poisonous to humans and livestock (Koiso *et al*., 1994; Li *et al*., 1995).

Conidia, thick-walled spores known as chlamydospores in this fungus, formed pleurogenetically on conidiophores that are formed on the floral organ of the rice (Brefeld, 1895; Bischoff *et al*., 2004; Tanaka *et al*., 2011) The chlamydospores germination can give rise to secondary conidia that can then produce tertiary conidia (Singh, 1984). The development of conidiospores and chlamydospores has been observed on synthetic medium (Fujita *et al*., 1989). The sclerotia of *V. virens* existing on or under the soil surface may germinate and form fruit-body (Singh and Dubey, 1984). It is thus clear that the fungus has several kinds of propagules. In order to observe more precisely and in greater detail than previous descriptions which known about the structure of each propagulum. The combined use of optical microscopy and scanning electron microscopy will provide a comprehensive understanding of the surface morphology of the fungus. Here, we present the development structure of propagules of *V. virens* isolated from infected rice kernels.

The advent of electrophoretic techniques for PFGE to separate the intact chromosomal DNA molecules of lower eukaryotes (Carle and Olson, 1984; Schwartz and Cantor, 1984), has provided a novel means of characterizing the chromosome sets of these organisms. This technique may be expected to provide fundamentally new information about the basic organization of the genomes of many species,

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particularly because numerous members of taxonomic groups including yeast (Carol and Olson, 1985), *Penicillium* (Rong *et al.*, 2010), *Mycosphaerella* (Mehrabi *et al.*, 2007), and *Rhizoctonia* (Keijer *et al.*, 1996) have proven intractable both to genetic and cytogenetic analysis. There has been a reproductive interaction between the genetic definition of genetic groups and the cytogenetic characterization of chromosomes as physical entities. Up to the present, little is known about the size or number of chromosomes in *V. virens*. Here, we report the application of the PFGE method to generate a molecular karyotype for this fungus.

Materials and Methods

Fungal isolates and culture conditions

Naturally infected rice kernels showing typical false smut symptoms were collected in a field plot at Wenjiang, China in 2010. The specimens were stored in dry envelopes at room temperature prior to the preparation for microscopy and morphological analysis.

The four *V. virens* isolates, SC01, SC06, SC09, and SC15 were used in electrophoretic karyotype study. The isolates were cultured in the XBZ solid medium consisting of 300 g/L potato, 5 g/L tryptone, 15 g/L sucrose, 0.5 g/L $Ca(NaO_3)_2 \cdot 4H_2O$, 2 g/L $Na_2HPO_4 \cdot 12H_2O$, and 1,000 ml H_2O. The solid medium was prepared by the addition of 1.5% (w/v) technical agarose. All of the reagents were of analytical grade and were obtained from Sigma-Aldrich (USA), except when mentioned otherwise. All of the isolates were individually maintained on each 9 cm diam. Petri dishes containing XBZ solid medium or in test slants at 4°C.

Preparation of chlamydospores and karyological observation

The chlamydospores of *V. virens* were obtained as a spore mass from cultures on XBZ solid medium and naturally infected rice kernels. A fresh chlamydospore suspension was cultivated in a preserving moisture level at 25°C for 2 d, during which the germination of the chlamydospores produced a large number of secondary conidia. The specimens prepared were stained with the DAPI nucleic acid stain, and the fixed specimens prepared using tuber burst methods were mounted in fluorescence antifading solution (Johnson and Araujo, 1981) containing 1 µg/ml DAPI and immediately observed by using fluorescence microscopy.

Preparation of specimens for scanning electron microscopy

The yellow chlamydospores groups and sclerotia of *V. virens* were fixed for 1 h in TBS (0.05 M Tris-HCl, 0.15 M NaCl, pH 7.0) containing 2.5% glutaraldehyde, rinsed twice in phosphate buffer (0.2 M, pH 7.2), and dehydrated in a graded series of alcohol for 10 min each, then prepared for critical point drying in carbon dioxide. The preparations were observed without metal coating although good results have been also obtained when the specimens were coated with a thin laver of gold, and scanned on the scanning electron microscopy screen (JSM-5900LV; JEOL Ltd., Japan) in order to obtain specimens size and surface morphology before recording micrographs.

Preparation of agarose-embedded DNA

Triangular flasks (150 ml) containing 50 ml of the XBZ liquid medium were inoculated with 10 small pieces each of 6×6×1 mm^3, crushed with forceps, cut from the edge of the XBZ solid cultures of the isolates. The flask cultures were incubated on a shaker at 28°C for 5 d. The hyphae of *V. virens* produced a large number of conidia in the XBZ liquid medium, and these conidia were harvested and washed twice with 50 µM EDTA, pH 8.0, at 4°C; the final conidium pellet was suspended in 50 µM EDTA, pH 8.0, and the final yield was determined with a hemocytometer. The concentration of conidia reached up to 2×10^8 ml^{-1}. A 400 µl aliquot of the conidiospore suspension was mixed at 50°C with an equal volume of 1.4% low melt agarose (prepared in 0.1 M EDTA; pH 8.0, 0.01 M Tirs-HCl; pH 7.6, and 0.02 M NaCl and equilibrate to 50°C), and then the mixture was transferred to plug molds using sterile transfer pipettes (Bio-Rad). The agarose was allowed to solidify at 4°C for 10–15 min, which added strength to the agarose for removal from the mold. The solidified agarose plugs were pushed into a 50 ml conical centrifuge tube containing 10 ml of a lyticase solution consisting of 10 mg/L lyticase, 0.6 M NaCl, 8.4 mM NaH_2PO_4, and 1.6 mM NaH_2PO_4. The plugs were incubated for 3 h at 37°C, removed from the lyticase solution and rinsed the plugs with sterile water. The resulting plugs were incubated in NDS [1% (w/v) lauroyl sarcosine, 0.5 M EDTA, and Tirs-HCl, pH 9.5] containing 2 mg/ml proteinase K (Sigma, USA) for 24 h twice at 50°C. The plugs were subsequently stored at 4°C in 0.5 M EDTA; pH 8.0. The plugs should be stable for 3 mo.

Pulsed field gel electrophoresis

PFGE was performed on a CHEF-DR II pulsed field system (Bio-Rad) (Chu *et al.*, 1986). The DNA molecules were separated in 0.6% Megabase agarose (Bio-Rad) at 12°C in 0.5× Tris-borate-EDTA buffer. The voltage, switching intervals, and total running times were as follows: 2.0 V/cm with a ramped 1200- to 1800-sec switching interval for 24 h, 48 h, 60 h, and 72 h. *Schizosaccharomyces pombe* strain 972h chromosomes (Bio-Rad, MW: 5.7, 4.6, and 3.5 Mb), *Saccharomyces cerevisiae* YNN295 chromosomes (Bio-Rad, MW: 2.2, 1.6, 1.125, 1.02, 0.945, 0.825, 0.785, 0.75, 0.68, 0.61, 0.565, 0.45, 0.365, 0.285, and 0.225 Mb) and the Trans 15k DNA marker (TransGen Biotech, MW: 15, 10, 7.5, 5, 3, and 1.5 kb) served as molecular weight markers. The gels were stained after the electrophoresis for 1 h with 1 µg/L ethidium bromide and destained for 1 h in ultrapure water then photographed with UV illumination.

Results

Morphological features of the *V. virens* colonies

The morphology in the early stages of the culture of *V. virens*, grown slowly on synthetic XBZ medium, resembled white bread (Fig. 1A). As the pathogen grew, the characteristics of the colony gradually varied and began to resemble a straw hat (Fig. 1B), the color of the colony became yellowish white. In the later stages, 40 days after the start of the culture,

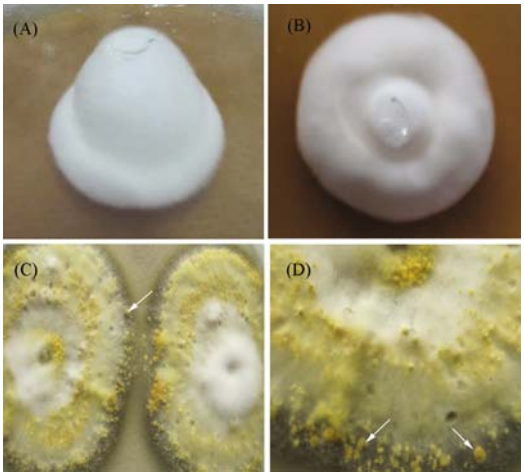

Fig. 1. Colonies of *V. virens* on the XBZ solid medium. (A) An isolate of *V. virens*, 10 d after inoculation. (B) The morphology of colony resembled a straw hat, 20 d after inoculation. (C–D) There were many mounds of chlamydospores (arrows) formed on the colony margin and a great number of chlamydospores dispersed on the whole colony, 40 d after inoculation.

a large number of yellowish chlamydospore balls formed on the colony, and the mature balls spilled out a great quantity of chlamydospores (Figs. 1C and 1D).

Karyological observation of conidia and hyphae

The chlamydospores produced in culture medium were prone to germinate in distilled water and produced secondary spores, the same as the fresh chlamydospores from naturally infected rice kernels. The conidia are holoblastically and sympodially produced at the apex of each conidiophore cells (Tanaka *et al*., 2011) (Fig. 2A). Karyological observation of DAPI stained nuclei was performed on conidia and hyphae. The development of the germ tubes resulted in the top of hyphae becoming mononuclear conidiospores (Fig. 2B). The secondary conidiospores sprouted hyphae, giving rise to multinucleate apical and basal cells (Figs. 2C and 2D).

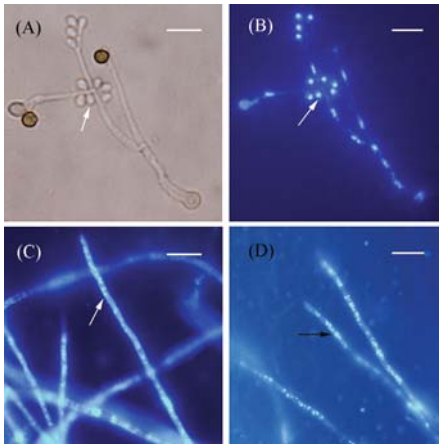

Fig. 2. Chlamydospores of germinated *V. virens* and karyological observation. (A) The conidia are holoblastically and sympodially produced at the apex of each conidiophore cells. (B) Conidia were mononuclear (arrows). (C–D) Somatic hyphae were multinuclear (arrows). Bars: A–D=10 μm.

Scanning electron microscopy features of the *V. virens* chlamydospores

The infected rice kernels were almost fully covered with a great quantity of *V. virens* chlamydospores (arrows) (Fig. 3A). Scanning electron microscopy revealed the irregularly round globose nature of the masses and the ornamented chlamydospores on the spore ball masses (Fig. 3B). The interiors of the false smut balls were intertwined with hyphae. Higher magnifications of the chlamydospores clearly showed prominent spore surface ornamentations, previously referred to as processed spines (Hashioka *et al*., 1966; Kim and Park, 2007) (Fig. 3C). The spines were pointed at the apex or irregularly curved and were approximately 250–550 nm long. The connections between the protruding spines were visible. A large number of chlamydospores massed on the surface of the chlamydospore balls that were grown on the XBZ solid medium (Fig. 3D). The chlamydospores were directly produced on the mycelium. An enlargement of the chla-

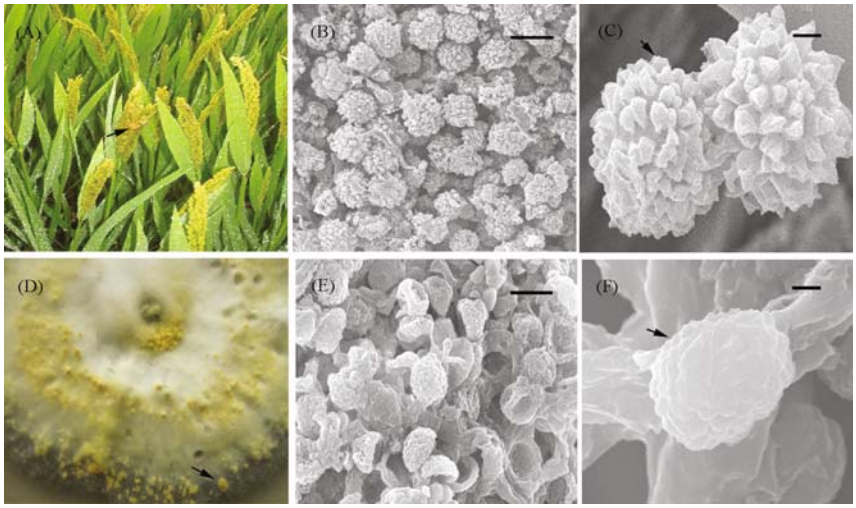

Fig. 3. Symptom of rice false smut and scanning electron micrographs of *V. virens* chlamydospores came from natural false smut balls and laboratorial culture. (A) A naturally infected rice kernel (arrows). (B) Chlamydospores masses. (C) Higher magnification of the chlamydospores. (D) A large number of chlamydospore balls piled on the XBZ solid medium. (E) Chlamydospores masses. (F) Higher magnification of a chlamydospore, with wavy spines (arrows) were prominent on the chlamydospores surface. Bars: B, E=10 μm. C, F=1 μm.

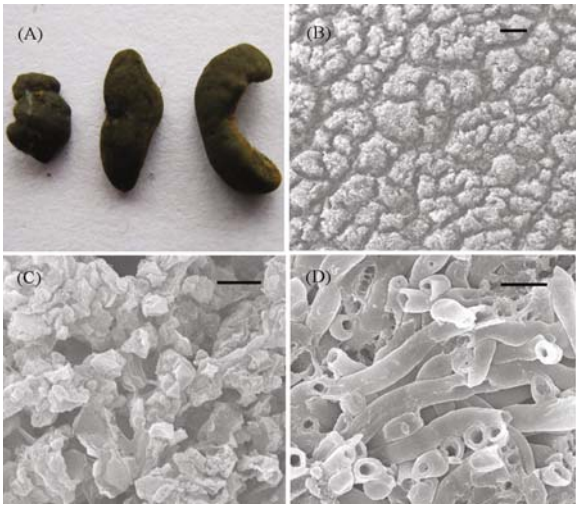

Fig. 4. Scanning electron micrographs of *V. virens* sclerotia. (A) Sclerotia of *V. virens*, which appeared black horseshoe-shaped and irregular oblong or flat, and sizes ranged from 2 to 20 mm. (B–C) Surface shape of a sclerotium. (D) Internal structure of a sclerotium, comprising dense mycelium. Bars: B=5 μm. C–D=10 μm.

mydospore clearly showed the arc-shaped spines (Figs. 3E and 3F). Obviously, the shape of the spines differed from the spines on the natural chlamydospores.

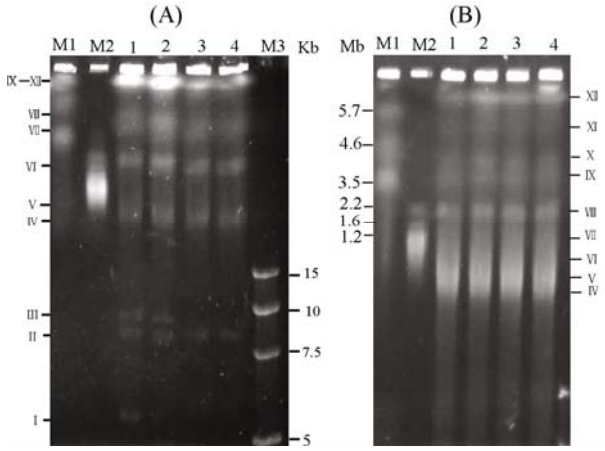

Fig. 5. The PFGE of *V. virens* isolates using a contour clamped homogeneous electric field (CHEF). The pulsed field gel shows the total DNA of *S. pombe* strain 972h (lane M1), S. *cerevisiae* strains YNN295 (lane M2), Trans 15K DNA marker (lane M3), and *V. virens* isolates SC01 (lane 1), SC06 (lane 2), SC09 (lane 3), and SC15 (lane 4). The PFGE was performed as described in the 'Materials and Methods' section except for the running times, which were 48 h (A), 72h (B).

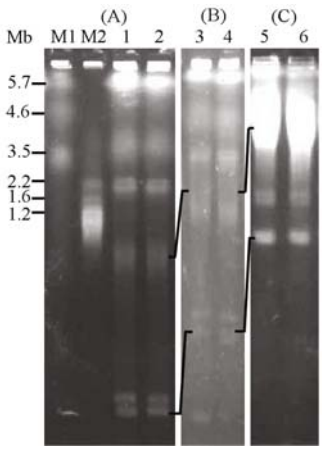

Fig. 6. Comparison the different running time (A, 60 h; B, 48 h; C, 24 h) for the electrophoretic karyotypes of *V. virens* strains SC01 and SC06. Pulsed field gel of total DNA of *S. pombe* strain 972 h (lane M1), *S. cerevisiae* strains YNN295 (lane M2) and *V. virens* isolates SC01 (lane 1, 3, 5) and SC06 (lane 2, 4, 6).

Scanning electron microscopy microstructure of *V. virens* sclerotia

The *V. virens* sclerotia collected in field were produced on the mature false smut balls, which appeared black horseshoe-shaped and irregular oblong or flat, and sizes ranged from 2 to 20 mm (Fig. 4A). As seen by scanning electron microstructure, the outer sclerotia wall appeared rough at a relatively low resolution (Fig. 4B). At a higher magnification, the projections made characteristic impressions in the sclerotia surface (Fig. 4C). The interior of each sclerotium was intertwined with compact hyphae (Fig. 4D).

Pulsed field gel electrophoresis

The electrophoretic karyotypes of the four isolates are shown in Fig. 5. By using different running time (Fig. 6), a sufficient resolution over the whole molecular weight range could be obtained to determine the number and size of the chromosomal bands that are present in *V. virens* (Table 1). From this, the total genome sizes were estimated. Isolates SC01, SC06, SC09, and SC15 have 12, 11, 10, and 10 chromosomes respectively, with genome sizes estimated at 23.5, 23.5, 23.5, and 23.6 Mb, respectively. Using a standard curve derived from the migration of the Trans 15 kb marker, the sizes of the three smallest chromosomes were estimated to be 6, 8, and 9 kb. The largest chromosome observed was approximately 6.0 Mb. The chromosome size was very similar to that observed for the yeast *S. pombe*. It is thus possible to estimate the sizes of *V. virens* chromosomes using the suggested sizes of the yeast chromosomes as a reference (Table 1). Despite the use of different electrophoretic conditions, the possibility cannot be excluded that one or more chromosomal bands remained unobserved due to co-mi-

Table 1. The molecular weights of the chromosomal bands of *V. virens*. The molecular weights of the chromosomal bands of *V. virens* isolates SC01, SC06, SC09, and SC15 are estimated on the basis of the given molecular weights of the molecular size standards.

Isolate	Size	Molecular weights of chromosomal bands in kb											
		I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
SC01	23499	6	8.5	9.5	200	225	750	1200	2200	3500	4000	5500	5900
SC06	23497		8	9	180	250	750	1200	2200	3500	4000	5500	5900
SC09	23488		8		180	250	750	1200	2200	3500	4000	5500	5900
SC15	23588		8		180	250	750	1200	2200	3500	4000	5500	6000

gration in the gel. The indicated number of chromosomes and genome sizes should therefore be considered as a minimum number.

Discussion

This study demonstrated the morphology of colonies and the structure of the *V. virens* propagules by the combined use of optical microscopy and scanning electron microscopy. A few characteristics were consistent with what has been previously reported (Bischoff *et al*., 2004; Chen *et al*., 2007; Kim and Park, 2007; Tanaka *et al*., 2008), but other characteristics have not been previously described. The morphological structures of conidia grown in liquid culture and artificial rearing chlamydospores have not been previously reported. The outer surface of the secondary conidia characterized by a smooth surface and thin wall, differed from the thick-walled spores. Secondary conidia can directly produce the next generation of the thin-walled conidia when they germinate, and this type of reproduction process could repeat successively. This special reproduction method was an important characteristic of *V. virens*. The conidiospores from the liquid culture created a mixture that should have many generations. Furthermore, the finding of our research that liquid culture can be used not only to produce conidia but also to form chlamydospores, differed from those previously reported (Zhang *et al*., 2003; Chen *et al*., 2007).

The microscopical observations of the *V. virens* nuclei in the conidia and somatic hyphae were done after staining the nuclei with the fluorescent dye DAPI (Taga and Murata, 1994). Our observations of the mitosis process allowed a measurement of the number of nuclei of *V. virens*. All previous studies of this fungus suggest that the chromosomes were very small and therefore difficult to count, after the number of chromosomes obtained by using PFGE. In this study, using fluorochromes that may be superior to the Feulgen, Giemsa, and aniline blue-glycerine dye that were used in previous studies, the nuclei were clearly observed. The conidia of *V. virens* were mononuclear, after germination of the hyphae that were multinuclear. This nuclear number transformation is a novel result in this fungus. The number of nuclei may be used as a classification standard of the fungus. For instance, the classification system of the *Rhizoctonia* species is based on the number of nuclei per cell in somatic hyphae (Flentje *et al*., 1963).

For appearance observations, scanning electron microscopy offered an integrated viewpoint of the fungal chlamydospore (Kim and Park, 2007). One of the morphological differences between *V. virens* chlamydospores and the smut teliospores of *Ustilaginaceae* was evident on the spore surface. The teliospores of *Neovossia horrida* have an outer sheath that obscures their spiny nature (Nawaz and Hess, 1987); however, *V. virens* chlamydospores were shown to have no such outer sheath in this study and the previously reported. This study is the first to identify the morphological differences between artificially reared and natural chlamydospores, which were evident on the spore surface. The natural chlamydospores had the sharp or irregular spines; however, the artificially reared chlamydospores were shown to have the smooth and regular spines. The structure of the natural chlamydospores may be the result from the long- term interaction between the pathogen and host, and may be more advantageous for the preliminary infection of the host. In contrast, the artificial XBZ rearing medium consisted of sufficient nutrition and may have led to gradual degeneration of the external structure of the chlamydospores.

This study is the first to report the sclerotium structure of *V. virens* observed by using scanning electron microscopy. The interior composition of the sclerotia were made of mycelium, therefore the sclerotia can germinate and form fruit-body, which can act as a preliminary infection source. Meanwhile, the germination of sclerotium can produce sexually reproductive stages (Singh and Dubey, 1984; Ou, 1985) along with a separation and restructuring of sexual reproduction. Therefore, the spores composing the sclerotia have more extensive genetic variation.

In this study, the application of different running conditions of PFGE facilitated the separation of the whole range of *V. virens* chromosomes spanning 6–6,000 kb, and we estimated the genome sizes to be at least 23.5 Mb. There is a high variation in the chromosome size among *V. virens.* This study is the first to estimate the chromosome number and genome size of *V. virens*, which will be helpful in both molecular and genetic studies, for example, in calculating the required size of genomic libraries or in the construction of genomic maps. Neither the genome size, the chromosome sizes, chromosome numbers nor the variation that is observed in these characteristics is unusual for filamentous fungi. Based on our experience with other filamentous fungi (Taga *et al*., 1998; Tsuchiya and Taga, 2001; Suga *et al*., 2002), as well as numerous reports of karyotyping of other filamentous fungi containing >3.9 Mb chromosome bands (Beadle *et al*., 2003) using CHEF, we concluded that the >4 Mb bands in this study represent individual chromosomes. The migration of the DNA molecules in a PFGE gel depends not only on their size but also on the number of DNA molecules to be separated in a well. In addition many other parameters can influence the migration, such as the buffer strength, concentration of agarose and running temperature (Beadle *et al*., 2003). Therefore, a direct comparison of our current data with that obtained by TAFE (McDonald and Martinez, 1991) is difficult. However, the use of the *S. pombe* and *S. cerevisiae* size markers in addition to Trans DNA 15 kb lends credibility to our size measurements and analyses.

This study indicated that *V. virens* has a unique characteristic karyotype. The genome comprises many small chromosomes (<0.2 Mb) but also large chromosomes (>4.0 Mb) including one chromosome that is ≥6 Mb; this finding is similar to other filamentous ascomycetes. The smallest chromosome we observed was 6 kb, and chromosomes II and III were smaller than 0.2 Mb (Table 1). Chromosomes ranging in size from 0.2 to 2 Mb are called supernumerary chromosomes or minichromosomes, which are common in plants and animals and have been reported in fungi as well (Skinner *et al*., 1991; Beadle *et al*., 2003). However, the small size of the *V. virens* chromosomes smaller than 0.2 Mb has not been reported in other filamentous ascomycetes. Another char-

acteristic of the *V. virens* genome is its chromosome length polymorphism (CLP). We estimated the chromosome length ranges from 0.6 to 6,000 kb. CLP has been reported within the asexual fungi of ascomycetes (Boehm *et al*., 1994). Chromosomal aberrations resulting in CLP may be generated by mitotic processes (Zolan, 1995). It has been proposed that asexual fungi may have a high degree of CLP, because chromosomal aberrations are more likely to persist in the absence of selection for homologous chromosomes during meiosis (Kistler and Miao, 1992). The asexual *Fusarium oysporun f.* sp. *Phaseoli* is reported to have a higher degree of CLP than the sexually mating population (Suga *et al*., 2002). Although the frequency of the sexual stage of *V. virens* in nature is unknown, meiosis could explain the low degree of CLP within a mating population.

The results of this study offered a powerful basis for further research. We know little of the morphological structure and growth process of the propagules of *V. virens*, and to date, we have not clearly identified the fungal infection process nor the primary infection source. Therefore, our results are useful for the study of the relationship between the pathogen and host, and provide theoretical basis for preventing this disease and determining the source of the primary infection. Moreover, the study of the molecular aspects of *V. virens* has progressed slowly. We still have not identified the virulence genes of *V. virens*, or their locus on a chromosome. The electrophoretic karyotype analysis presented here estimated the number of chromosomes and the genomic size of *V. virens,* which will be helpful in both molecular and genetic studies.

Acknowledgements

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