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Cytological and electrophoretic karyotyping of the chestnut blight fungus *Cryphonectria parasitica*

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ABSTRACT

The karyotypes of nine strains including three transformants of the chestnut blight fungus *Cryphonectria parasitica* were analyzed by pulsed-field gel electrophoresis (PFGE) and cytology using a fluorescence microscope. Cytology of the mitotic metaphase showed $n = 9$ for both standard strain EP155 and field strain GH2 infected by *Cryphonectria hypovirus 3*. Chromosomes were morphologically characterized by size, heterochromatic segment, and constriction. PFGE resolved 5 or 6 chromosomal DNA bands ranging from 3.3 Mbp to 9.7 Mbp, but accurate determination of the chromosome number was hampered by clumping of some bands. Banding profiles in PFGE were similar among the strains except for GH2, in which a chromosome translocation was detected by Southern blot analysis. By integrating the data from cytology and PFGE, the genome size of *C. parasitica* was estimated to be *ca.* 50 Mbp. This is the first report of a cytological karyotype in the order Diaporthales.

1. Introduction

The ascomycete *Cryphonectria parasitica*, a member of the family Cryphonectriaceae in the order Diaporthales (Gryzenhout et al., 2006), is the causal pathogen of chestnut blight disease. It was introduced to the United States and Europe in the 19th and 20th centuries from east Asia, most probably from Japan (Griffin and Elkins, 1986; Milgroom et al., 1996), resulting in serious outbreaks in those areas. Billions of chestnut trees have been destroyed in the United States alone since the 19th century (Anagnostakis, 1982; Griffin and Elkins, 1986). Thus, chestnut blight is regarded as one of the three most devastating tree diseases in the world along with Dutch elm disease and white pine blister rust (Tainter and Baker, 1996).

C. parasitica has also attracted interest as a natural host of at least five virus families, some of which attenuate the virulence of the host fungus (Hillman and Suzuki, 2004). The fungal strains infected by the viruses have been studied intensively, aiming to develop a biological method for control of chestnut blight. Virus-infected hypovirulent fungal strains can convert virulent strains to hypovirulence by transferring their viruses via hyphal anastomosis (Anagnostakis, 1982). This property has contributed to the successful control of chestnut blight disease in Europe by artificially and naturally disseminating hypovirulent strains as a biological control agent (Heiniger and Rigling, 1994; Milgroom and Cortesi, 2004). In addition to such exploitation for practical purposes, *C. parasitica*/virus parasitic systems serve as a model for studying the molecular mechanisms of interactions between eukaryotic viruses and hosts (Nuss, 2005). The essential techniques for the molecular biology of *C. parasitica*, including transformation (Choi and Nuss, 1992; Churchill et al., 1990), targeted gene disruption (Gao and Nuss, 1996; Kasahara et al., 2000; Lan et al., 2008), and artificial inoculation of viruses (Chen et al., 1994; Choi and Nuss, 1992; Hillman et al., 2004), have already been developed. Using these techniques and a publicly available EST library of the fungus (http://cogeme.ex.ac.uk/), viral/host factors and signal pathways in the fungus–virus interactions have been analyzed (Allen et al., 2003; Craven et al., 1993; Dawe and Nuss, 2001; Faruk et al., 2008a,b; Sun et al., 2006; Suzuki et al., 2003).

In spite of the importance of *C. parasitica* both as a plant pathogen and as a model organism, the genome of this fungus is poorly understood, especially in terms of its karyotype. So far, only one report has been published on the karyotype of *C. parasitica* (Milgroom et al., 1992), in which the chromosome number (CN) was estimated solely by pulsed-field gel electrophoresis (PFGE) with no information on the sizes of the chromosomes and genome.

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Recently, a linkage map was constructed incorporating three types of molecular markers and vegetative incompatibility (*vic*) loci (Kubisiak and Milgroom, 2006), but the map consists of 24 linkage groups and apparently connotes too many gaps to provide information on the chromosome complements. Despite the demand for karyotypic information for performing various genetic analyses, our understanding of the *C. parasitica* genome is still in its infancy.

The main purpose of this study was to determine the karyotype of *C. parasitica* by combining cytological and electrophoretic karyotyping. Most importantly, the standard wild type strain EP155, whose genome has just been sequenced and temporarily assembled (http://genome.jgi-psf.org/Crypa1/Crypa1.info.html) at the US Department of Energy – Joint Genome Institute (JGI), and the hypovirulent strain GH2, which has been used for more than 20 years as a representative host of hypovirus CHV3-GH2 (Hillman et al., 2000, 2004), were intensively analyzed so that our data contribute to both the genome project and the genetic analysis of *Cryphonectria*/virus interactions. In addition to the karyotype, chromosome polymorphism was also analyzed by electrophoretic karyotyping of the additional strains.

2. Materials and methods

2.1. Fungal strains and culture techniques

Six field strains and three transformants that originated from field strain EP155 were used. Transformants were included to check the occurrence of chromosome rearrangements caused by transformation. Their hosts, geographic origin, virus infection, and virulence toward apple are listed in Table 1. Strains were stored in regeneration medium (Churchill et al., 1990) at 4 °C, and grown on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI) for routine use. Conidial suspensions for cytology and pulsed-field gel electrophoresis (PFGE) were prepared as follows: small pieces of mycelial agar blocks from a 5- to 7-d-old culture were inoculated onto PDA plates in 9 cm-diam plastic Petri dishes and incubated at 27 °C with continuous lighting to produce pycnidia. Conidia oozing from pycnidia were harvested from 1- to 3-wk-old cultures and placed in distilled water containing 0.02% (v/v) Tween-20 with a glass spreader. After being sieved through two layers of nylon mesh (Miracloth, 22–25 μm pore size, Calbiochem, La Jolla, CA) to remove mycelial debris, conidia were pelleted and washed twice with distilled water by centrifugation for 1–2 min at 3500 *g*. The final conidial pellet was suspended in potato dextrose broth (PDB) (Difco) to use for cytology and PFGE.

2.2. Cytological observation

Mitotic chromosomes of cytological specimens were prepared by modifying protocols of the germ tube burst method (GTBM) used for other fungi (Shirane et al., 1989; Taga and Murata, 1994; Taga et al., 1998). Eighty to one-hundred fifty microliters of a conidial suspension of *C. parasitica* in PDB (5×10^5–1×10^6 conidia/ml) was placed on a slide glass and incubated at 23 °C in a humid chamber for 36–40 h. One hour before the end of incubation, thiabendazole (Sigma, St. Louis, MO; stock solution: 10 mg/ml dimethyl sulfoxide) was added to the conidial suspension at a final concentration of 50 μg/ml. After incubation, slides were briefly dipped in distilled water to wash off PDB, wiped with filter paper around the area of the specimen, and immersed in fixative [methanol:glacial acetic acid = 9:1 (v/v)] for 15–30 min. Finally, the slides were flame-dried and were stored in dry condition until use.

For fluorescence staining of chromosomes, slides were mounted with an antifade mounting solution (Johnson and Araujo, 1981) containing 4′,6-diamidino-2-phenylindole (DAPI) alone at 1 μg/ml or both DAPI and propidium iodide (PI) at 1 μg/ml each (hereinafter, this staining is referred to as DAPI/PI staining). DAPI binds to DNA in an AT-specific manner, while PI binds to DNA with little or no sequence preference. Although DAPI alone can visualize chromosomes, DAPI/PI was additionally used expecting AT-rich regions to show up more clearly than DAPI alone. Furthermore, chromosome banding to highlight AT-rich region was attempted by treating preparations with a GC-specific nonfluorescent antibiotic actinomycin D (AMD; Wako Pure Chemical, Osaka, Japan) prior to DAPI staining (hereinafter, referred to as AMD/DAPI staining). To perform AMD/DAPI staining, the procedures of Schweizer (1976) were used with modifications. Briefly, slides were first flooded with AMD dissolved at 0.2 mg/ml in McIlvaine buffer (pH 7) for 15 min, rinsed with McIlvaine buffer, and air-dried at room temperature. Then, the slides were stained with DAPI as above.

Observations were made by UV-excitation using an epifloures-cence microscope (Olympus BH2/BHS-RFC or Nikon Eclipse E600/Y-FL) equipped with a 100× oil immersion objective lens (N.A. = 1.3). Fluorescent images were recorded using an Olympus digital camera (C5050-Z or DP-70,) attached to the microscope

Table 1
Strains of *Cryphonectria parasitica* used in this study.

Strain	Host/locality	Virulence to apple[a]	Virus infection[b]	Source/reference
EP155 (=ATCC38755)	American chestnut/Connecticut, USA	+++	Virus-free	D.L. Nuss/Hillman et al. (1990)
EP67 (=ATCC38753)	European chestnut/Italy	++	Virus-free	B.I. Hillman/ Anagnostakis (1978)
NB58 (=ATCC76220)	American chestnut/New Jersey, USA	–	Infected with CHV2-NB58	B.I. Hillman/Hillman et al. (1992)
GH2	American chestnut/Michigan, USA	+	Infected with CHV3-GH2	B.I. Hillman/Hillman et al. (2000)
9B21	American chestnut/West Virginia, USA	–	Infected with MyRV1-Cp9B21	B.I. Hillman/Hillman et al. (2004)
MAFF 410878	Japanese chestnut/Japan	+	Virus-free	MAFF
AC1	Transformant of EP155	+++	Incorporating partial length cDNA of CHV1 mutant Cys(70) and *hph*[c]	N. Suzuki/Eusebio-Cope (2007)
nam*A*	Transformant of EP155	–	Incorporating full length cDNA of CHV1 mutant Cys(72) and *hph*[c]	N. Suzuki/Faruk et al. (2008a,b)
AC2/Δp69	Transformant of EP155	–	Infected with Δp69 and incorporating *hph*[c]	N. Suzuki/Eusebio-Cope (2007)

[a] Data from Eusebio-Cope (2007) +++: highly virulent, ++: moderately virulent, +: weakly virulent, –: very weakly or non-virulent (hypovirulent).
[b] For detailed description of viruses, refer to Hillman and Suzuki (2004).
[c] *hph:Escherichia coli* hygromycin B phosphotransferase gene.