Mycologia, 102(6), 2010, pp. 1295–1302. DOI: 10.3852/10-040

Genome size variation in the pine fusiform rust pathogen *Cronartium quercuum* f.sp. *fusiforme* as determined by flow cytometry

Claire L. Anderson
School of Forest Resources and Conservation, P.O. Box 110410, University of Florida, Gainesville, Florida 32611

Thomas L. Kubisiak[1]
C. Dana Nelson
USDA Forest Service, Southern Research Station, Southern Institute of Forest Genetics, 23332 Old Mississippi 67, Saucier, Mississippi 39574

Jason A. Smith
John M. Davis
School of Forest Resources and Conservation, P.O. Box 110410, University of Florida, Gainesville, Florida 32611

Abstract: The genome size of the pine fusiform rust pathogen *Cronartium quercuum* f.sp. *fusiforme* (Cqf) was determined by flow cytometric analysis of propidium iodide-stained, intact haploid pycniospores with haploid spores of two genetically well characterized fungal species, *Sclerotinia sclerotiorum* and *Puccinia graminis* f.sp. *tritici*, as size standards. The Cqf haploid genome was estimated at ~90 Mb, similar to other Pucciniales species for which reference genome sequences are available. Twenty-three Cqf pycniospore samples were compared that comprised three samples obtained from naturally occurring pine galls and 20 samples obtained after artificial inoculation with parental isolates and their progeny. Significant variation in genome size (>10% of mean) was detected among unrelated as well as sibling Cqf samples. The unexpected plasticity in Cqf genome size observed among sibling samples is likely to be driven by meiosis between parental genomes that differ in size.

Key words: *Cronartium quercuum* f.sp. *fusiforme*, flow cytometry, fusiform rust disease, genome size, propidium iodide, pycniospore, spermatia

INTRODUCTION

Cronartium quercuum (Berk.) Miyabe ex Shirai f.sp. *fusiforme* (Cqf) is a heteroecious, macrocyclic fungal pathogen that infects red oak and hard pine species and is the causal agent of fusiform rust disease. Cqf is endemic to the southeastern United States and is the most economically damaging fungal disease of pine trees in this region (Anderson et al. 1986). Genetic resistance to Cqf is a priority for loblolly (*Pinus tadea* L.) and slash (*Pinus elliottii* var. *elliottii* Engelm.) pine breeding programs (Bridgwater et al. 2005, Byram et al. 2006), and considerable progress in selecting rust-resistant pines has been made. Resistance is often conferred gene-for-gene (Flor 1971) in which the disease interaction is dictated by the presence or absence of single genes for resistance in the host and specifically corresponding genes for avirulence in the pathogen. At least nine fusiform rust (*Fr*) resistance genes have been identified and mapped in loblolly pine with genetic markers (H. Amerson pers comm, Jordan 1997, Wilcox et al. 1996). However little is known about the corresponding avirulence (*Avr*) genes present in the pathogen. Our research aims to identify these genes (Nelson et al. 2010) and to develop molecular tools for Cqf that will let *Avr* gene frequencies be monitored in natural populations. This would allow pine plantation managers to deploy rust-resistant genotypes in the most effective manner, thereby reducing losses to fusiform rust disease and helping to secure pine as a biomass feedstock, carbon sequestration tool and a source of high quality renewable raw materials.

An accurate estimate of Cqf genome size is required as a first step toward developing a reference genome sequence that would aid map-based cloning of *Avr* genes. Flow cytometry has become the method of choice to determine the size of plant and animal genomes because it is high throughput sensitive and requires minimal sample preparation (Doležel et al. 2007, Kron et al. 2007) and a number of studies have applied this method to the examination of fungal species (Almeida et al. 2007, Carr and Shearer 1998, Dvorak et al. 1987, Eilam et al. 1994, Gourmet et al. 1997, Hirij and Sanders 2004, Kim et al. 2000, Kullman 2000, O'Sullivan et al. 1998, Stöver et al. 1998, Yeater et al. 2002). In this process whole cells or isolated nuclei are stained with a fluorescent DNA-binding dye and particles in suspension are hydrodynamically focused into a single-file stream as they pass a light source that excites the dye, allowing light scattering and fluorescence data to be individually collected for each particle in the sample.

The sample of interest must be compared to a reference sample of known DNA content to accurate-

Submitted 23 Feb 2010; accepted for publication 25 Apr 2010.
[1]Corresponding author. USDA Forest Service, Southern Research Station, Southern Institute of Forest Genetics, 23332 Old Mississippi 67, Saucier, MS 39574. E-mail: tkubisiak@fs.fed.us

ly determine genome size by flow cytometry. Chicken red blood cells or nuclei are commonly used in plant and animal studies. However because the chicken genome is ~2.5 pg or ~2445 Mb (Rasch et al. 1971, Tiersch et al. 1989) and most fungal genomes that have been studied to date are 10–60 Mb (Gregory et al. 2007) chicken nuclei are too large to be suitable size standards in fungal flow cytometry experiments. Haploid and diploid *Saccharomyces cerevisiae* strains have been used as alternative size standards in studies of small fungal genomes (Almeida et al. 2007, Carr and Shearer 1998, Hirij and Sanders 2004, Kullman 2000). However the ~12 Mb yeast genome (Goffeau et al. 1996) may be too small to use as a standard for plant pathogenic rust fungi, which tend to have larger genomes of ~100 Mb or more (http://www.broadinstitute.org/annotation/genome/puccinia_group/MultiHome.html; http://genome.jgi-psf.org/Mellp1/Mellp1.info.html).

The aim of this study was to determine the haploid Cqf genome size among unrelated and sibling Cqf pycniospore samples. Haploid spermatia of *Sclerotinia sclerotiorum* isolate 1980 and haploid pycniospores of *P. graminis* f.sp. *tritici* isolate CRL 75-36-700-3 were used as size standards because these two fungal species have been genetically well characterized and reference genome sequences are available. Significant genome size variation was observed among unrelated isolates as well as among sibling pycniospore samples of Cqf, suggesting that intraspecific genome size variation is common in natural populations of this rust pathogen.

MATERIALS AND METHODS

Sources of fungal material.—Haploid pycniospores of *Cronartium quercuum* f.sp. *fusiforme* (Cqf) were obtained from three naturally occurring galls on 18 mo old loblolly pine rooted cuttings that were infected in Raleigh, North Carolina, and maintained in a greenhouse (University of Florida, Gainesville). The pycniospore samples were named according to the identification number of the pine cutting from which they were collected (137B, 689B and 644A respectively). Numerous pycnia droplets were collected and pooled from each gall to generate single-gall pycnia collections that were stored at −20 C until use. The three single-gall pycnia collections are likely to contain genetically pure populations of pycniospores because research has shown that natural infection generally (>95%) results from infection by a single basidiospore (Kubisiak et al. 2004). Parental and sibling samples of Cqf pycniospores were obtained by artificial inoculation of 8 wk old open-pollinated progenies of loblolly pine family 10-5 with basidiospores of isolates NC2-40, SC20-21 (Kuhlman and Matthews 1993) or P2. P2 is a single uredinia pustule-derived line that was produced from a cross between NC2-40 and SC20-21, the details of which will be described elsewhere (T. Kubisiak in prep). Inoculations were performed at the U.S. Forest Service Resistance Screening Center in Asheville, North Carolina, with the concentrated basidiospore spray system (Matthews and Rowan 1972). Inoculated seedlings were transported to the University of Florida and maintained in a greenhouse until sporulation. Individual pycnia droplets were harvested from galled trees and stored separately at −20 C until use. Care was taken to avoid mixing between droplets because research has shown that the galls produced after artificial inoculation often result from infection by more than one basidiospore and that in such cases genetic variability might exist between the droplets produced (Kubisiak et al. 2004).

Haploid *Sclerotinia sclerotiorum* (Ss) spermatia were provided by Jeffrey Rollins (Department of Plant Pathology, University of Florida) and were obtained from a spermatia-overproducing mutant of the Ss wild-type isolate 1980. Plate cultures grown on artificial medium (http://www.sclerotia.org/) that had produced numerous sclerotia were flooded with 10 mL sterile water to produce a spermatia suspension. The suspension was recovered from the plate and sterile glycerol was added to a final concentration of 30% (v/v) before storage at −80 C. Haploid *Puccinia graminis* f.sp. *tritici* (Pgt) pycniospores were obtained from Les Szabo (USDA ARS Cereal Disease Laboratory, University of Minnesota) after inoculation of barberry (*Berberis* spp.) with basidiospores of Pgt isolate CRL 75-36-700-3. Pycnia droplets were pooled and stored at −80 C until use.

Propidium iodide staining and flow cytometry.—All spores were centrifuged 1 min at 16 000 *g*, and any supernatant was removed. Unfixed spores were resuspended in TENT buffer (10 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 mM NaCl, 0.1% (v/v) Triton X-100), and the concentration was determined with a Neubauer hemacytometer. Spores were stained in 0.5 mL TENT buffer that contained 10 µg/mL propidium iodide (PI; Sigma-Aldrich, St Louis, Missouri), 1 mg/mL DNase-free RNase A (QIAGEN, Valencia, California) and 200 000 spores/mL. Samples were incubated at room temperature in the dark on a vertical rotator at approximately 20 rpm up to 72 h.

Samples were analyzed with a BD LSR II flow cytometer (BD BioSciences, San Jose, California) equipped with a solid state 100 mW 488 nm argon laser. PI fluorescence was acquired with a 610/20 nm band pass filter, and data were displayed with a logarithmic scale. Forward scatter (FSC) and side scatter (SSC) data were acquired with a 488/10 nm band pass filter, and data were displayed with linear scales. Data for 10 000 events were collected with BD FACSDiva 6.0 software (BD BioSciences, San Jose, California) and 1–9 replicate samples were analysed for each fungal sample and treatment. Data analysis was performed with FCS Express 3 software (De Novo Software, Los Angeles, California). Histograms of DNA-binding induced PI fluorescence were produced with a logarithmic scale for PI fluorescence and a linear scale for number of events. Histogram markers that encompassed the entire peak of fluorescence were used to calculate the median and coefficient of variation (CV) for each sample.

Confocal fluorescence microscopy.—Spores were imaged with a TCS SP5 laser scanning confocal microscope (Leica

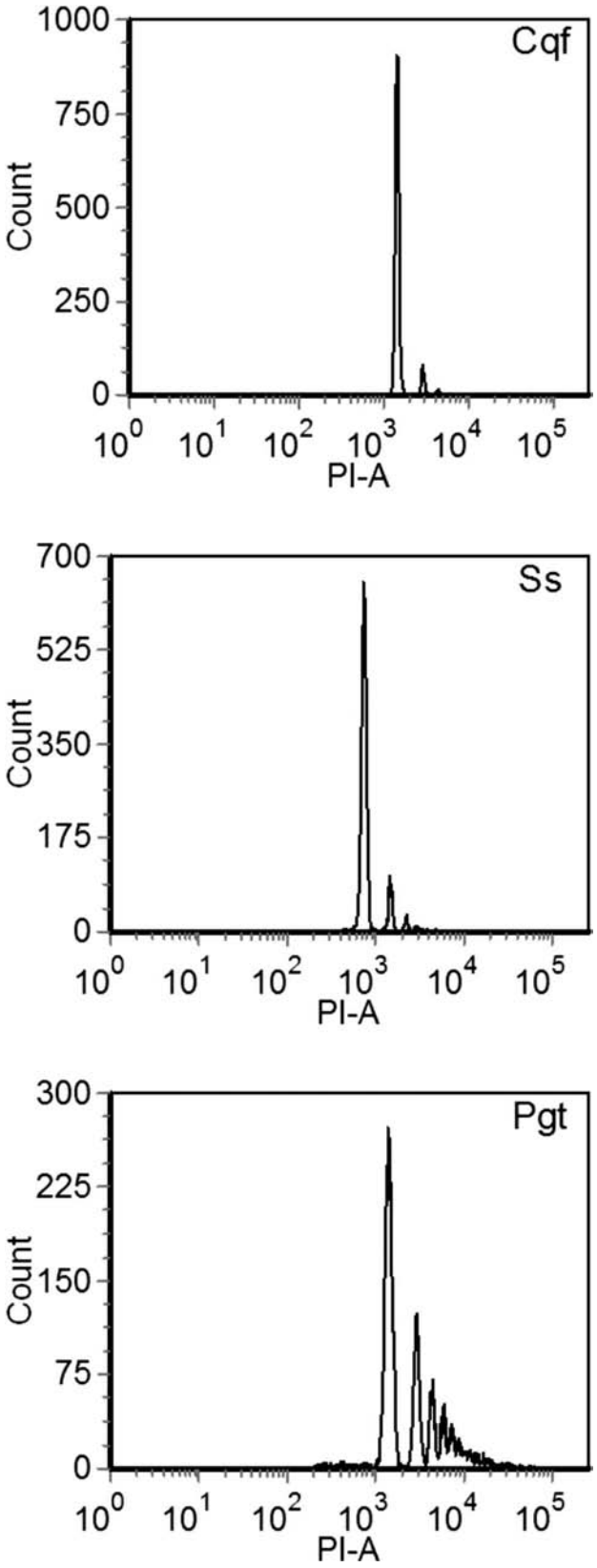

FIG. 1. DNA binding-induced propidium iodide (PI) fluorescence histograms produced after flow cytometric analysis of Cqf, SS and Pgt spores. DNA binding-induced PI fluorescence values are given in arbitrary units. All spore samples were incubated in buffer containing PI 48 h with addition of RNase A 4 h before analysis.

Microsystems Inc., Bannockburn, Illinois) and either 63× or 100× oil immersion objective. A 488 nm laser line was used for excitation, and the PI signal was collected in an acoustic-optical beam splitter window of 550–700 nm. Leica LAS-AF 2.01 software (Leica Microsystems Inc., Bannockburn, Illinois) was used for instrument control and image analysis.

Statistical means tests.—Tukey's studentized range tests were performed on PI fluorescence and the corresponding estimated genome sizes. Mean differences were declared significant at $P \leq 0.05$.

RESULTS

Optimizing spore preparation procedures for flow cytometry.—To optimize propidium iodide (PI) staining of Cqf pycniospores, Pgt pycniospores and Ss spermatia a time-course experiment was conducted in which spore samples were incubated with PI for 0–72 h. RNase A was added to all PI-stained samples 4 h before analysis, irrespective of the length of incubation with PI, and the entire experiment was repeated three times.

Flow cytometric analysis of unstained samples indicated that none of the spore types displayed autofluorescence (data not shown). However clearly defined, major peaks of fluorescence were observed in PI fluorescence histograms for all samples that had been stained with PI (FIG. 1). Dot plots of forward scatter (FSC) against side scatter (SSC) indicated that both Pgt and Cqf pycniospores were of similar size and texture whereas Ss spermatia were larger as indicated by greater FSC values (data not shown). In these dot plots Cqf pycniospores and Ss spermatia formed clustered populations indicating that spores were of uniform size and shape. In PI fluorescence histograms for Cqf pycniospores and Ss spermatia the major peak often was accompanied by a minor peak of approximately double the fluorescence intensity that is likely to represent spore doublets (FIG. 1). Pgt pycniospores formed the most dispersed population in dot plots of FSC against SSC (data not shown), and in PI fluorescence histograms the major peak was accompanied by a series of additional peaks of increasing fluorescence intensity but diminishing prevalence (FIG. 1). It is likely that these additional peaks represent pairs and groups of spores because Pgt pycniospores appeared to be of uniform size and shape when examined by light microscopy but had a

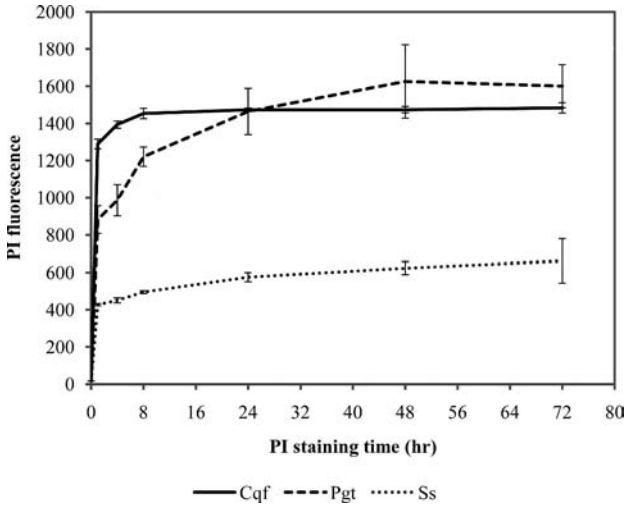

FIG. 2. The effect of staining time on DNA binding-induced propidium iodide (PI) fluorescence. Spore samples were incubated in buffer containing PI 0–72 h and RNase A was added to all samples 4 h before analysis. The data lines represent the mean and error bars represent ± standard deviation obtained from three independent experiments. DNA binding-induced PI fluorescence is given in arbitrary units.

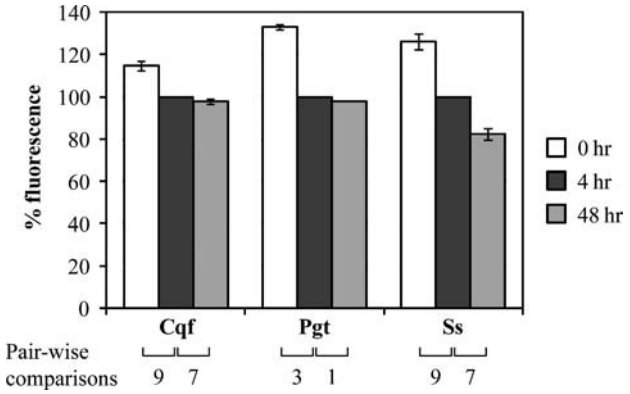

FIG. 3. The effect of RNase A incubation time on DNA binding-induced propidium iodide (PI) fluorescence. All spore samples were incubated with PI 48 h before analysis. RNase A was added either 4 or 48 h before analysis or was omitted (0 h). The histograms represent the mean relative amount of PI fluorescence, normalized with the fluorescence of corresponding samples that were treated with RNase A 4 h in a pairwise comparison. Error bars represent ± standard deviation. The number of pairwise comparisons made between treatments is indicated.

greater propensity than Cqf pycniospores or Ss spermatia to form aggregates (data not shown).

For all three spore types, dot plots of FSC against PI fluorescence indicated that DNA content was uniform despite some variation in spore size (data not shown). These characteristics were observed repeatedly in the PI-staining time-course experiments and in all subsequent experiments. However DNA binding-induced PI fluorescence increased with increasing PI incubation time and maximal staining was achieved after 48 h for all three spore types (FIG. 2). Therefore 48 h was chosen as the PI incubation period for all further experiments.

Because PI is a nucleic acid intercalating dye that does not discriminate between DNA and RNA an additional experiment was conducted to determine the effect of RNase A incubation time on PI fluorescence. Spore samples were stained with PI for 48 h and RNAse A was added 4 h or 48 h before analysis or was omitted. Incubation with RNase A for 4 h was sufficient to remove almost all RNA from Cqf and Pgt pycniospores (FIG. 3). However for Ss spermatia incubation with RNase A for 48 h caused a 17.61 ± 2.65% reduction in PI fluorescence compared to the 4 h RNase A treatment (FIG. 3). Therefore when using Pgt as a reference all samples were treated with RNase A for 4 h before analysis and when using Ss spermatia as a reference all samples were treated with RNAse A 48 h.

None of the three spore types exhibited significant nonspecific PI staining when examined by confocal fluorescence microscopy (FIG. 4). Cqf pycniospores were subpyriform and approximately 2.5 × 3.5 μm; a number of vesicles were observed (FIG. 4B) and the nucleus appeared to be elongated, in accordance with the observations of Mims and Doudrick (1996). Intense staining of the Cqf nucleus was observed and no obvious difference was seen between the fluorescence of Cqf pycniospores treated with RNase A for 4 r (data not shown) or 48 h (FIG. 4A–C). Pgt pycniospores were similar in size and shape to Cqf pycniospores and also possessed an elongated nucleus that stained brightly and exclusively with PI (data not shown). In contrast Ss spermatia were spherical and were approximately 3.5 μm diam. Each spermatium contained one large vesicle and a single spherical nucleus, in accordance with the characteristics reported by Willetts (1997). The nucleus stained brightly and exclusively with PI. No observable difference was seen between the fluorescence of Ss spermatia treated with RNase A for 4 h (data not shown) or 48 h (FIG. 4D–F), indicating that visual examination of spores by fluorescence microscopy alone was insufficient to detect the change in RNA content detected by flow cytometry upon prolonged incubation with RNase A.

Cqf genome size estimation.—Cqf genome size was determined with three Cqf single-gall pycniospore collections from naturally infected pine trees (137B, 689B and 644A) and 20 individual pycnial droplets

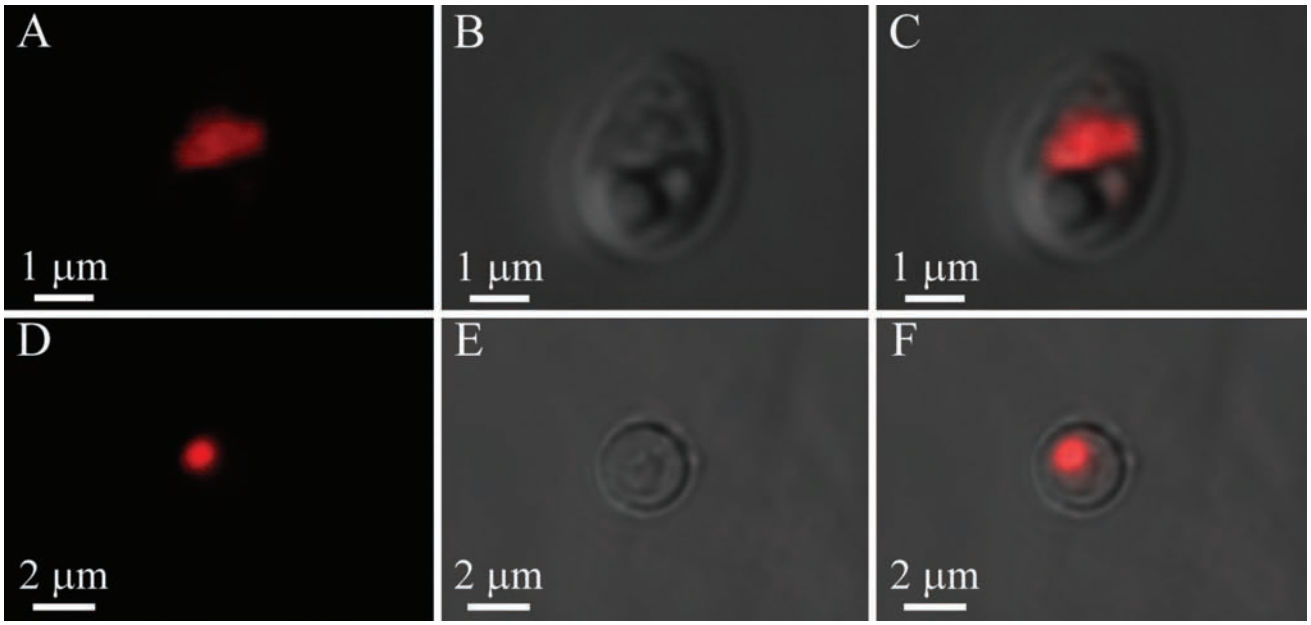

FIG. 4. Examination of propidium iodide (PI)-stained spores by confocal microscopy. Cqf137B pycniospores (panels A–C) and Ss spermatia (panels D–F) were stained with PI and treated with RNase A 48 h before analysis. Spores were viewed by confocal microscopy with a 100× oil immersion objective. A, D. PI fluorescence. B, E. Bright field. C, F. Merged bright field/PI fluorescence.

collected from galled pine trees of the open-pollinated family 10-5 that had been artificially inoculated with isolate NC2-40 (five sibling samples, NC2-40-1–NC2-40-5), SC20-21 (five sibling samples, SC20-21-1–SC20-21-5) or P2 (10 sibling samples; P2-1–P2-10). Pgt pycniospores and Ss spermatia were used as size standards, and the entire experiment was replicated four times.

When using Pgt pycniospores as reference the calculated haploid genome sizes of the 23 Cqf pycniospore samples ranged from 82.57 ± 2.25 Mb for P2-8 to 93.35 ± 2.09 Mb for Cqf644A (FIG. 5; SUPPLEMENTAL TABLE I), assuming a Pgt genome size of 88.64 Mb as indicated by genome sequencing efforts (http://www.broadinstitute.org/annotation/genome/puccinia_group/MultiHome.html). No significant differences were found between the genome sizes estimated for the three pycniospore collections arising from the same location in Raleigh, North Carolina (Cqf137B, 644A and 689B). However these three samples had significantly larger genomes than several of the individual NC2-40, SC20-21 and P2 samples (FIG. 5). No significant differences were observed among the five NC2-40 sibling samples or the five SC20-21 sibling samples, but significant differences were detected among the 10 P2 sibling samples (FIG. 5). When the mean genome sizes of isolates NC2-40, SC20-21 and P2 were determined by averaging across the representative sibling sets (SUPPLEMENTAL TABLE I) NC2-40 had the smallest genome of the three isolates examined at 85.26 ± 2.70 Mb. The mean genome sizes of isolates P2 and SC20-21 (88.43 ± 3.47 Mb and 88.24 ± 3.19 Mb respectively) were not significantly different from one another but were significantly larger than NC2-40 as determined by both Duncan's and Tukey's means tests ($P < 0.05$).

When using Ss spermatia as reference the genome sizes calculated for the 23 Cqf pycniospore samples ranged from 88.58 ± 1.60 Mb for P2-8 to 98.21 ± 2.17 Mb for Cqf644A (FIG. 5; SUPPLEMENTAL TABLE I), assuming a Ss genome size of 38.33 Mb as indicated by genome sequencing efforts (http://www.broadinstitute.org/annotation/genome/sclerotinia_sclerotiorum/MultiHome.html). Although use of Ss spermatia as reference led to slightly larger estimates of Cqf genome size than had been calculated with Pgt, similar patterns of variation were observed. No significant differences were observed among the five NC2-40 or five SC20-21 sibling samples, but significant differences were observed among the 10 P2 sibling samples and the mean genome size of isolate NC2-40 was significantly smaller than the mean genome sizes of isolates SC20-21 and P2 as determined by both Duncan's and Tukey's means tests ($P < 0.05$).

DISCUSSION

The main goal of this study was to determine the genome size of the pine fusiform rust pathogen *Cronartium quercuum* f.sp. *fusiforme* (Cqf) by flow

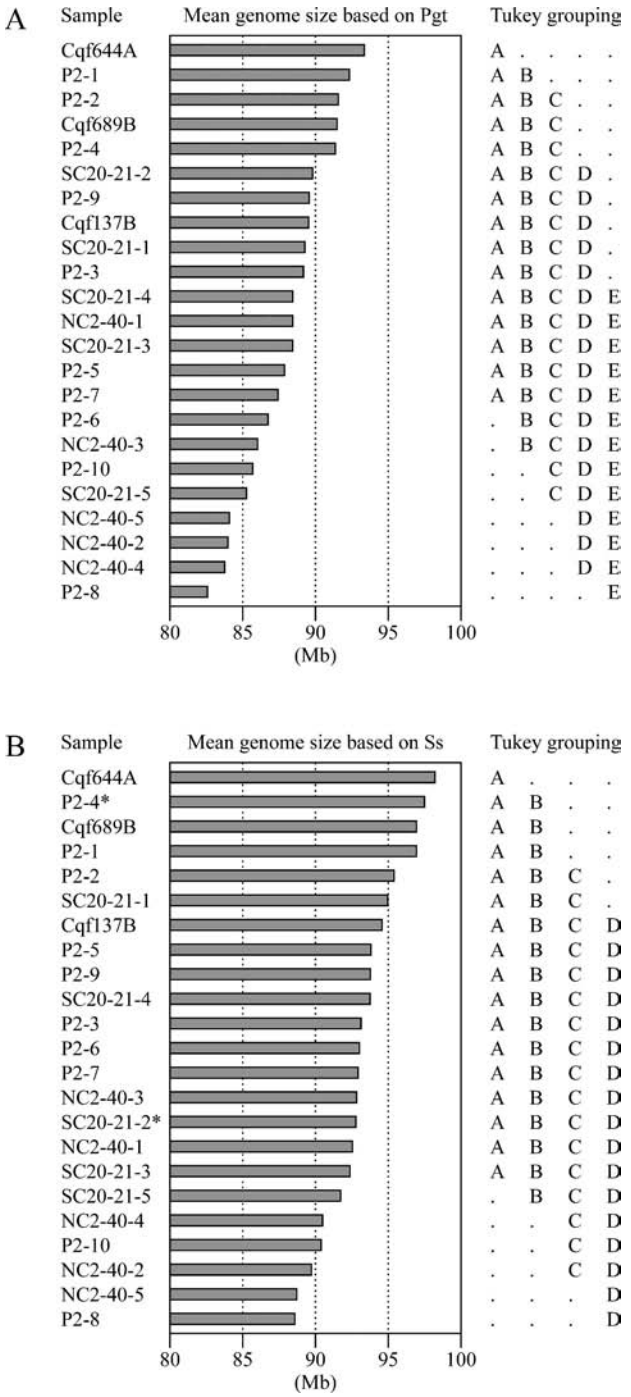

FIG. 5. Mean genome sizes of Cqf samples as determined with Pgt pycniospores or Ss spermatia as reference. Mean genome size was calculated for each of the Cqf samples after four replicate experiments, except where indicated by an asterisk that denotes only three replicate experiments were performed. The dataset was subjected to analysis using Tukey's studentized range test ($P < 0.05$). Samples with the same uppercase letters were not significantly different. A. All spores were incubated with PI 48 h and RNase A 4 h before analysis and Cqf genome size was calculated with Pgt pycniospores as a size standard. B. All spores were incubated with PI and RNase A 48 h before analysis and Cqf genome size was calculated with Ss spermatia as a size standard.

cytometry with two fungal species, *Puccinia graminis* f.sp. *tritici* (Pgt) and *Sclerotinia sclerotiorum* (Ss), as standards. Upon determination of the optimal parameters for sample preparation, the genome size of 23 Cqf pycniospore samples were 82.57–93.35 Mb with Pgt as reference or 88.58–98.21 Mb with Ss spermatia as reference. Taken together these data indicate a genome size of approximately 90 Mb for Cqf, confirming that the pine fusiform rust pathogen has a genome that is larger than most fungal pathogens analyzed to date (Gregory et al. 2007) but similar to two other plant pathogenic rust pathogens, *Puccinia graminis* f.sp. *tritici* (88.64 Mb; http://www.broadinstitute.org/annotation/genome/puccinia_group/MultiHome.html) and *Melampsora larici-populina* (101.1 Mb; http://genome.jgi-psf.org/Mellp1/Mellp1.info.html), for which reference genome sequences are available.

Although use of Ss spermatia as reference generally led to slightly larger genome size estimates than obtained with Pgt pycniospores, the mean difference between estimates for the 23 Cqf samples tested was just 5.17 ± 1.04 Mb. Given that the two sets of Cqf genome estimates were obtained in independent experiments, this suggests that the data obtained are reliable despite the fact that neither *S. sclerotiorum* nor *P. graminis* have been used as size standards in published flow cytometry studies. In contrast similar experiments performed with PI-stained, commercially prepared chicken erythrocyte nuclei lead to an overestimation of Pgt and Ss genome size with calculated means of 164.32 ± 5.54 Mb and 83.66 ± 3.58 Mb respectively (assuming a chicken genome size of 2445 Mb, C. Anderson unpubl). Therefore our data supports proposals by Eilam et al. (1994) and Kullman (2000) that the large chicken genome makes it unfavorable for use as a reference in flow cytometry studies involving much smaller fungal genomes.

The small discrepancies observed between the Pgt-based and Ss-based genome size estimates for the 23 Cqf pycniospore samples could have been caused by factors such as minor contributions to PI fluorescence by residual RNA, nonspecific staining, mitochondrial DNA or quenching of fluorescence by secondary metabolites (Dvorak et al. 1987, Greilhuber 2007, Lemke et al. 1978). However another source of error in flow cytometry studies is the accuracy of the genome size estimate for the reference standard itself. The genome sequence of *Sclerotinia sclerotiorum*

has been fully assembled and is likely to be complete (J. Rollins pers comm), but gaps remain in the *Puccinia graminis* f.sp. *tritici* sequence assembly and the current estimate of 88.64 Mb is likely to be an underestimate of Pgt genome size (L. Szabo pers comm). This would result in an underestimate of Cqf genome size with Pgt as the standard and might explain why Pgt-based estimates of Cqf genome size were smaller that those calculated with Ss spermatia as reference. Limited quantities of Pgt pycniospores unfortunately were available for use in this project because they are difficult to produce by artificial inoculation and cannot be cultured outside the host plant. Therefore it was not possible to calculate the genome size of Pgt directly with Ss spermatia as reference. However when the mean genome sizes of the 23 Cqf samples are used as a conversion factor an indirect estimate of Pgt genome size based on Ss spermatia is 93.86 ± 1.14 Mb.

Our analysis of 23 pycniospore samples has provided the first indication that genome size variation exists in *Cronartium quercuum* f.sp. *fusiforme*, with a 10.78 Mb (12.2% of average) or 9.63 Mb (10.3% of average) range in genome size observed when Pgt pycniospores or Ss spermatia were used respectively as size standards. Genome size variation is commonly observed among fungal species and can result from chromosome length polymorphisms (CLPs) or gain/loss of complete chromosomes (CNPs) (reviewed in Zolan 1995). It will be interesting to determine whether CLPs or CNPs are responsible for the significant size variation observed here among Cqf samples. Although the actual mechanism responsible for the differences observed in this study can only be speculated, the significant difference in genome sizes observed between the two parental isolates, NC2-40 and SC20-21, combined with the continuous nature of the observed size differences for sibling P2 samples, suggest that CLPs are a more likely mechanism. Studies based on SSRs (Burdine et al. 2007, Kubisiak et al. 2004) and the presence of numerous codominant RAPDs and AFLPs observed in genetic mapping (Kubisiak unpubl data) suggest that Cqf harbors size variation at specific genetic marker loci, but the magnitude of genome size variation observed in this study, particularly among sibling samples, was not anticipated.

The main goal of this work was to determine the size of the Cqf genome because this information is invaluable for map-based cloning and when planning a complete genome sequencing project. We recently mapped a single Avr gene (*Avr1*) in *Cqf* and will be employing map-based strategies to clone this gene (manuscript in prep). The Cqf genome sequence will be obtained in 2010 through the Joint Genome Institute (JGI) Community Sequencing Program (http://www.jgi.doe.gov/sequencing/cspseqplans2010.html). The sequence will be prepared with haploid DNA extracted from a genetically pure Cqf pycniospore sample to greatly facilitate sequence assembly. Genome size for the sample submitted to JGI for sequencing will be determined by flow cytometric analysis to provide an estimate of the anticipated final assembly. Nevertheless repetitive sequences, such as those in centromeric or telomeric regions, are often not fully represented in sequence assemblies (cf. Bennett et al. 2003). Therefore it is likely that the completed genome sequence will differ slightly in size to that estimated by flow cytometry.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the contributions of Tania Quesada (School of Forest Resources and Conservation, University of Florida), Charles Barnes and Les Szabo (USDA ARS Cereal Disease Laboratory, University of Minnesota) and Jeffrey Rollins (Department of Plant Pathology, University of Florida) for providing fungal material, without which this project could not have been completed. We also thank Katherine Smith (USDA-Forest Service, School of Forest Resources and Conservation, University of Florida) for excellent technical assistance, Neal Benson (Interdisciplinary Center for Biotechnology Research Flow Cytometry Core Lab (ICBR-FCCL), University of Florida) for providing advice and assistance with flow cytometry and Steve McLellan (ICBR-FCCL, University of Florida) for providing confocal fluorescence microscopy services. The ICBR-FCCL is financially supported by an equipment grant from the Bankhead-Coley Cancer Research Program. This research was supported in part by Cooperative Agreement 09-CA-11330126-058 between the USDA-Forest Service Southern Research Station (Southern Institute of Forest Genetics) and the University of Florida.

LITERATURE CITED

Almeida AJ, Matute DR, Carmona JA, Martins M, Torres I, McEwen JG, Restrepo A, Leão C, Ludovico P, Rodrigues F. 2007. Genome size and ploidy of *Paracoccidioides brasiliensis* reveals a haploid DNA content: flow cytometry and GP43 sequence analysis. Fung Genet Biol 44:25–31.

Anderson RL, McClure JP, Cost N, Uhler RJ. 1986. Estimating fusiform rust losses in five southeast states. South J Appl For 10:237–240.

Bennett MD, Leitch IJ, Price HJ, Johnston JS. 2003. Comparisons with *Caenorhabditis* (~100 Mb) and *Drosophila* (~175 Mb) using flow cytometry show genome size in *Arabidopsis* to be ~157 Mb and thus ~25% larger than the Arabidopsis Genome Initiative estimate of ~125 Mb. Ann Bot 91:547–557.

Bridgwater F, Kubisiak T, Byram T, McKeand S. 2005. Risk assessment with current deployment strategies for

fusiform rust-resistant loblolly and slash pines. South J Appl For 29:80–87.

Burdine CS, Kubisiak TL, Johnson GN, Nelson CD. 2007. Fifty-two polymorphic microsatellite loci in the rust fungus, *Cronartium quercuum* f. sp. *fusiforme*. Mol Ecol Notes 7:1005–1008.

Byram TD, Miller LG, Raley EM. 2006. 54th Progress Report of the Western Gulf Forest Tree Improvement Program. College Station: Texas A&M Univ Press. p 1–28.

Carr J, Shearer G. 1998. Genome size, complexity, and ploidy of the pathogenic fungus *Histoplasma capsulatum*. J Bacteriol 180:6697–6703.

Doležel J, Greilhuber J, Suda J. 2007. Estimation of nuclear DNA content in plants using flow cytometry. Nature Protocols 2:2233–2244.

Dvorak JA, Whelan WL, McDaniel JP, Gibson CC, Kwon-Chung KJ. 1987. Flow cytometric analysis of the DNA synthetic cycle of *Candida* species. Infect Immun 55: 1490–1497.

Eilam T, Bushnell WR, Anikster Y. 1994. Relative nuclear DNA content of rust fungi estimated by flow cytometry of propidium iodide-stained pycniospores. Phytopathology 84:728–735.

Flor HH. 1971. Current status of the gene-for-gene concept. Annu Rev Phytopathol 9:275–276.

Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H, Oliver SG. 1996. Life with 6000 genes. Science 274:546–567.

Gourmet C, Gray LE, Rayburn AL. 1997. Flow cytometric analysis of conidia of fungi isolated from soybean vascular tissue. J Phytopathol 145:405–408.

Gregory TR, Nicol JA, Tamm H, Kullman B, Kullman K, Leitch IJ, Murray BG, Kapraun DF, Greilhuber J, Bennett MD. 2007. Eukaryotic genome size databases. Nucl Acids Res 35:D332–D338.

Greilhuber J. 2007. Cytochemistry and C-values: the less well known world of nuclear DNA amounts. Ann Bot (London) 6:791–804.

Hirij M, Sanders IR. 2004. The arbuscular mycorrhizal fungus *Glomus intraradices* is haploid and has a small genome size in the lower limit of eukaryotes. Fung Genet Biol 41:253–261.

Jordan AP. 1997. Fusiform rust disease resistance and genomic mapping in loblolly pine [Master's thesis]. Raleigh: North Carolina State University. 105 p.

Kim M-S, Klopfenstein NB, McDonald GI, Arumuganathan K, Vidaver AK. 2000. Characterization of North American *Armillaria* species by nuclear DNA content and RFLP analysis. Mycologia 92:874–883.

Kron P, Suda J, Husband BC. 2007. Applications of flow cytometry to evolutionary and population biology. Annu Rev Ecol Evol Syst 38:847–876.

Kubisiak TL, Roberds JH, Spaine PC, Doudrick RL. 2004. Microsatellite DNA suggests regional structure in the fusiform rust fungus *Cronartium quercuum* f. sp. *fusiforme*. Heredity 92:41–50.

Kuhlman EG, Matthews FR. 1993. Variation in virulence among single-aeciospore isolates from single-gall isolates of *Cronartium quercuum* f. sp. *fusiforme*. Can J For Res 23:67–71.

Kullman B. 2000. Application of flow cytometry for the measurement of nuclear DNA content in fungi. Folia Cryptog Estonica Fasc 36:31–46.

Lemke PA, Kugelman B, Morimoto H, Jacobs EC, Ellison JR. 1978. Fluorescent staining of fungal nuclei with a benzimidazol derivative. J Cell Sci 29:77–84.

Matthews FR, Rowan J. 1972. An improved method for large-scale inoculations of pine and oak with *Cronartium fusiforme*. Plant Dis Rep 56:931–934.

Mims CW, Doudrick RL. 1996. Ultrastructure of spermatia and spermatium ontogeny in the rust fungus *Cronartium quercuum* f. sp. *fusiforme*. Can J Bot 74:1050–1057.

Nelson CD, Kubisiak TL, Amerson HV. 2010. Unravelling and managing fusiform rust disease: a model approach for coevolved forest tree pathosystems. For Pathol 40: 67–72.

O'Sullivan D, Tosi P, Creusot F, Cooke M, Phan T-H, Dron M, Langin T. 1998. Variation in genome organisation of the plant pathogenic fungus *Colletotrichum lindemuthianum*. Curr Genet 33:291–298.

Rasch EM, Barr HJ, Rasch RW. 1971. The DNA content of sperm of *Drosophila melanogaster*. Chromosoma 33:1–18.

Stöver AG, Witek-Janusek L, Mathews HL. 1998. A method for flow cytometric analysis of *Candida albicans* DNA. J Microbiol Methods 33:191–196.

Tiersch TR, Chandler RW, Wachtel SS, Elias S. 1989. Reference standards for flow cytometry and application in comparative studies of nuclear DNA content. Cytometry 10:706–710.

Wilcox PL, Amerson HV, Kuhlman EG, Lui B-H, O'Malley DM, Sederoff RR. 1996. Detection of a major gene for resistance to fusiform rust disease in loblolly pine by genomic mapping. Proc Natl Acad Sci USA 93:3859–3864.

Willetts HJ. 1997. Morphology, development and evolution of stromata/sclerotia and macroconidia of the Sclerotiniaceae. Mycol Res 101:939–952.

Yeater KM, Grau CR, Rayburn AL. 2002. Flow cytometric analysis of *Phialophora gregata* isolated from soybean plants resistant and susceptible to brown stem rot. J Phytopathology 150:258–262.

Zolan ME. 1995. Chromosome-length polymorphisms in fungi. Microbiol Rev 59:686–698.