Detection of DNA Polymorphisms in a Single Urediniospore-Derived Culture of *Cronartium quercuum* f. sp. *fusiforme*

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ABSTRACT

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PCR (polymerase chain reaction) with single, arbitrarily chosen primers was used to detect DNA polymorphisms among 34 sibling gametothalli (single-basidiospore hyphal cultures) and spermatia (single drops of pycniospores) derived from a single-urediniospore culture of the fusiform rust fungus, *Cronartium quercuum* f. sp. *fusiforme*. Ten single-basidiospore hyphal cultures were distinguished by comparing nine polymorphic segments produced by four primers. Polymorphisms also were detected between 24 single drops of pycniospores collected from four different galls and drops within the same gall, revealing that phenotypes of galls were all different, and some contained more than one phenotype. The possibility of the presence of more than one genotype between and within galls reinforces a belief that traditional experiments on the genetics of host-pathogen interactions between *Pinus* and *C. q. fusiforme* cannot be carried out using current methods of inoculation. The ability to identify genetic variation within a single urediniospore-derived culture of *C. q. fusiforme* should benefit efforts to minimize heterogeneity in populations of basidiospores used in artificial inoculations. The polymorphisms can be applied in studies of population variation, used for genetic mapping, and used in resolving specifics of sexuality and the ploidy level of various stages of the life cycle.

Fusiform rust disease, caused by *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme*, is the most important disease in pine forests (loblolly pine, *Pinus taeda* L., and slash pine, *P. elliottii* Engelm.) of the southeastern United States. Control measures have focused primarily on the development of planting stock through selection of open-pollinated families of *Pinus* spp. that exhibit general resistance to composites of aeciospore collections of *C. q. fusiforme*. Unfortunately, this approach provides little information on the genetics of host-pathogen interactions.

Variation in pathogenicity among aeciospores collected from several species of pine has been demonstrated with inoculation studies (13,16). However, precise genetic analyses have been restricted by limited knowledge of the reproductive biology of *C.*

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q. fusiforme and by the lack of genetic markers. Differentiation between and among *Cronartium* spp. has been evaluated by serological comparison, but no intraspecific differences were detected (6). Isozyme analyses on mass collections of aeciospores showed differences among formae speciales of *C. quercuum* but did not detect variation between single-gall collections of *C. q. fusiforme* (12,14).

Welsh and McClelland (20) and Williams et al (22) independently developed a method of identifying DNA polymorphisms. The method uses single, arbitrarily chosen primers in a polymerase chain reaction (PCR). The random amplified polymorphic DNA (RAPD) markers typically produced are inherited as dominant alleles of Mendelian loci. Each primer gives a different pattern of RAPD markers with the potential of detecting one or more polymorphic loci in a segregating population. The amount of genomic DNA required for assay is much less than that needed for other analyses, e.g., RFLP (restriction fragment length polymorphism). This advantage may be particularly important in studies of organisms like *C. q. fusiforme* for which the amount of available DNA is limited.

The primary objective of this study was to examine genetic variation in a single urediniospore-derived culture of *C. q. fusiforme* detected by RAPD markers. Hyphal cultures grown from basidiospores obtained from a single telial column provided template DNA used to screen primers and identify techniques that gave repeatable amplification. A subset of the primers was used to evaluate polymorphisms among single drops of pycniospores collected from fusiform rust galls produced by inoculation of loblolly pine seedlings with basidiospores from the single urediniospore-derived culture.

MATERIALS AND METHODS

The single urediniospore-derived culture (WLP-10-2.SS1) of *C. q. fusiforme* used in this study was started from aeciospores collected by G. A. Snow, during 1984, from a loblolly pine tree growing in Livingston Parish, near Walker, LA. A modification of the procedure for obtaining single aeciospore-derived cultures of *C. q. fusiforme* outlined by Powers (11) was used to produce the single urediniospore-derived culture. Uredinia were induced on a seedling water oak (*Quercus nigra* L.) inoculated with aeciospores from the Livingston Parish collection site. Fresh urediniospores were dusted onto the surface of water agar. A single spore was selected under a dissecting microscope and was transferred onto the adaxial surface of a young, succulent water oak leaf. The inoculated oak seedling was misted with water, enclosed in a plastic bag, and placed in a dark chamber for 24 h at 20 C. After incubation, the seedling was held in constant light at 20 C for 2 wk. Several water oak seedlings were inoculated with derivative urediniospores, increasing quantities of urediniospores for genetic analyses and storage. Throughout the study, oak seedlings were kept isolated from sources of contaminating *C. q. fusiforme* spores to maintain the purity of the single urediniospore-derived culture. Urediniospores from the single-urediniospore culture and from the original collection of aeciospores were dried and stored under vacuum at 4 C using the procedures of Roncadori and Matthews (15).

A single telial column on an inoculated water oak leaf was used to produce 10 single-basidiospore hyphal cultures. Preparation of the aqueous inoculum containing basidiospores followed the Method II procedures of Amerson et al (1). The basidiospore-germination medium was modified Harvey and Grasham basal medium (7) amended per liter with 0.1 g of $CaCO_3$, 1 g of yeast extract and peptone, and 10 g of bovine serum albumin (HGYP + BSA) (8). Nurse culture and single-basidiospore isolation techniques used to obtain the cultures were described by Hu and Amerson (8). Basidiospore germlings remained on HGYP + BSA at 21 C in a dark chamber for up to 2 mo. The resulting hyphal cultures were transferred to and maintained on modified Gresshoff and Doy 1 agar medium (18) amended by replacing glucose with sucrose and by adding per liter 1 g of yeast extract and peptone (GDYPS) (2). Stationary liquid cultures using GDYPS with agar omitted were grown at 21 C in a dark chamber to provide material for preparation and analysis of genomic DNA.

A forced air system (17) calibrated and maintained at 12–18 spores per square millimeter was used to inoculate 9-wk-old loblolly pine seedlings with basidiospores obtained from the single urediniospore-derived culture. Those seedlings that developed fusiform rust galls and abundant pycniospore production subsequently were retained for the study. At least six separate single drops of pycniospores (0.5–5 μl of pycniospores and 5–50 μl of nectar per drop) were collected from galls on four pine seedlings. Collected drops were distributed uniformly, no less than 1 cm apart, around the galls. The drops were harvested with an air-displacement pipette using filter-plugged microliter tips, were ejected into 1.2 M sorbitol, and were stored at −70 C.

Genomic DNA of single-basidiospore hyphal cultures and of single drops of pycniospores were prepared by a modified procedure combining protocols outlined by Geisen (5) and Lee and Taylor (9). The grinding/lysis buffer contained 50 mM Tris-HCl (pH 7.2), 50 mM EDTA (pH 8.0), and 3% SDS (sodium dodecyl sulfate) autoclaved for 15 min. One percent 2-mercaptoethanol was added after autoclaving. Spores (10–100 μl) or coarsely fragmented mycelium (100–500 μl) was combined 1:1 (v/v) with diatomaceous earth (Celite-diatomaceous earth; Sigma, St. Louis, MO) and 100–500 μl of grinding/lysis buffer. Samples were ground using a Pellet pestle (Kimble/Owens-Illinois, Vineland, NJ) operated with an electric drill at 1,200 rpm. The slurry resulting from grinding was spun at 800 *g*. After centrifugation, preparations were incubated at 65 C for 1 h.

Preparations were extracted twice with volumes equal to that of the preparation, first with phenol/chloroform/isoamyl alcohol 25:24:1 (v/v/v) saturated with 10 mM Tris (pH 8.0) and 1 mM EDTA and second with chloroform/1-octanol 24:1 (v/v). Each extraction mixture was vortexed lightly and very briefly and was spun at 10,000 *g* for 15 min. The extracted DNA was precipitated with 2–2.5 volumes of absolute ethanol and 0.1 final volume of 3 M sodium acetate (pH 5.2) equal to a final concentration of 200–300 mM. The precipitate was pelleted, rinsed with 70% ethanol, and dried under vacuum at least 30 min. The dried DNA pellet was reconstituted in TE buffer (10 mM Tris-HCl [pH 7.6] and 0.1 mM EDTA [pH 8.0]. For preparations containing significant amounts of RNA (estimated by gel electrophoresis of preparations), 1 μl of 10 mg/ml of RNase A (Sigma, St. Louis, MO) per 20 μl was added to the TE buffer at reconstitution. Preparations treated with RNase were reprecipitated.

Amplification of polymorphic segments of genomic DNA was carried out by modifying the methods outlined by Welsh and McClelland (20), Welsh et al (21), and Williams et al (22). Amplification reactions were carried out in 25 μl volumes containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.9 mM $MgCl_2$, 0.001%

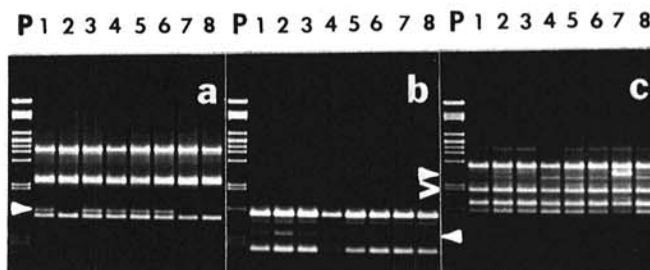

Fig. 1. Amplification, by three primers, of segments of genomic DNA from eight (lanes 1–8) single-basidiospore hyphal cultures of *Cronartium quercuum* f. sp. *fusiforme* derived from a single-urediniospore culture. **a, b, and c,** show segments amplified by primers OPD-11, OPE-11, and OPE-07, respectively. Amplified segments polymorphic between single-basidiospore hyphal cultures are marked by a solid arrow. The open arrow points to a segment monomorphic for the single-basidiospore hyphal cultures but polymorphic between the single drops of pycniospores. The amplified segments were resolved by electrophoresis through 2% agarose gels and were visualized by ethidium bromide staining. Size markers (P) are from *PstI* (I. B. I., New Haven, CT) restriction digestion of bacteriophage lambda (New England Biolabs, Beverly, MA).

gelatin, 100 μM each of dATP, dCTP, dGTP, and dTTP, 0.2 μM primer, 25 ng of denatured genomic DNA, and 1 unit of *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, IN). A set of 107 primers was chosen from OLIGO 10-mer kits A, B, D, E, F, and G, 20 primers each (Operon Technologies, Alameda, CA). Amplification was performed in a GeneAmp PCR system 9600 (Perkin-Elmer/Cetus, Norwalk, CT). Primer and dNTPs were added to the reaction mix in a modified Hot Start (Perkin-Elmer/Cetus) at 50 instead of 70 C (4). The DNA thermal cycler was operated through one preliminary cycle of 95 C for 1 min followed by 28 C for 5 min and 95 C for 1 min. This temperature profile was followed by forty cycles of 94 C for 20 s, 35 C for 1 min, and 72 C for 1 min, using the fastest available transitions between each temperature, followed by 7 min at 72 C. Amplification products were resolved by electrophoresis (5 v/cm) for 2 h in 2% A-6013 (type I: low EEO) agarose gels (Sigma) in 40 mM Tris base, 20 mM sodium acetate, 1 mM EDTA, and glacial acetic acid (pH 7.2) TAE buffer and were detected by staining with ethidium bromide.

RESULTS

Patterns of amplified segments of genomic DNA for single-basidiospore hyphal cultures. Genomic DNA for eight of the 10 single-basidiospore hyphal cultures was used in single-primer reactions to screen the set of 107 primers for amplification of polymorphic segments. Reaction combinations producing segments tentatively identified as polymorphic in the first phase of screening were replicated at least twice for verification. Figure 1 shows segments of genomic DNA amplified, using three primers, for eight single-basidiospore hyphal cultures. For the 107 primers screened, 370 segments were amplified for an average of approximately 3.4 segments per primer screened. However, most amplified segments were apparently monomorphic between cultures for each primer; 40 primers produced patterns identical for all eight cultures tested. Thirty-two primers produced weak or erratic patterns; 14 primers did not produce any product. Twenty-one primers amplified 32 reproducible polymorphic segments. Multiple polymorphic segments were produced by seven primers (Table 1).

The differences between patterns for the eight single-basidiospore hyphal cultures were used diagnostically to differentiate specific cultures. Four primers (OPA-11, OPB-10, OPB-18, and OPE-07) producing a total of nine polymorphic segments distinguished all eight cultures from each other (Table 1). Using these four primers, the two remaining single-basidiospore hyphal cultures were distinguishable from each other and from the eight cultures used in screening.

Patterns of polymorphic markers among single drops of pycniospores. Six drops of pycniospores collected from different regions on each of four galls were tested with the four primers (OPA-11, OPB-10, OPB-18, and OPE-07) that distinguished the 10 single-basidiospore hyphal cultures. Figure 2 shows segments of genomic DNA amplified from 24 separate, single drops of pycniospores collected from the four fusiform rust galls. Analysis of the patterns of amplified segments from the four galls showed that no patterns were the same between the galls. Within one gall, the patterns were the same for all drops. In contrast, different phenotypes were distinguished within the other three galls. Two different patterns of amplified segments were observed in two of these galls, and three patterns were observed in the third (Table 2).

TABLE 1. Patterns of polymorphic segments of DNA amplified with single primers using polymerase chain reaction (PCR) on eight single-basiodiospore hyphal cultures obtained as germinating basidiospores from a single-urediniospore infection of *Cronartium quercuum* f. sp. *fusiforme* on water oak

		PCR on single-basidiospore hyphal cultures[b]							
Primer (sequence)	Segment[a]	1	2	3	4	5	6	7	8
OPA-11 (CAATCGCCGT)	1,600	−	+	−	−	+	+	+	−
	800	+	−	+	+	+	−	+	+
OPB-04 (GGACTGGAGT)	900	−	+	−	+	+	+	+	+
	810	−	−	−	−	+	+	+	−
	450	+	−	+	−	−	−	+	+
OPB-10 (CTGCTGGGAC)	850	−	+	−	+	+	+	−	−
	805	−	+	−	+	−	−	−	−
	625	−	−	−	+	+	+	+	+
	340	−	−	−	+	+	+	+	−
OPB-18 (CCACAGCAGT)	1,500	−	−	−	−	−	−	−	+
	900	+	−	−	−	−	−	−	−
OPD-08 (GTGTGCCCCCA)	850	−	+	−	+	+	+	−	−
OPD-11 (AGCGCCATTG)	800	+	−	+	+	+	+	−	−
OPD-16 (AGGGCGTAAG)	550	−	+	−	−	−	−	−	+
OPE-01 (CCCAAGGTCC)	1,225	+	−	+	−	+	+	−	−
OPE-02 (GGTGCGGGAA)	700	−	+	−	+	+	+	−	+
OPE-05 (TCAGGGAGGT)	300	−	+	−	+	−	−	−	+
OPE-07 (AGATGCAGCC)[c]	1,400	−	−	−	−	−	−	+	−
OPE-11 (GAGTCTCAGG)	525	+	+	+	−	−	−	+	+
OPE-16 (GGTGACTGTG)	1,350	−	−	−	−	+	+	+	−
	1,200	−	−	−	+	+	+	+	−
OPE-18 (GGACTGCAGA)	1,500	+	+	+	−	+	+	+	−
	1,325	+	−	+	−	−	−	−	−
OPE-20 (AACGGTGACC)	800	+	+	+	+	+	+	+	−
OPF-02 (GAGGATCCCT)	500	−	+	−	+	−	−	−	−
OPF-04 (GGTGATCAGG)	1,050	−	+	−	+	+	+	−	−
	1,000	−	+	−	+	+	+	−	−
	950	+	−	+	−	−	−	+	+
OPF-14 (TGCTGCAGGT)	1,150	−	−	−	+	−	−	−	−
OPF-16 (GGAGTACTGG)	700	+	+	+	−	−	−	−	−
OPG-03 (GAGCCCTCCA)	825	−	+	−	−	+	+	+	−
OPG-08 (TCACGTCCAC)	850	−	+	−	−	+	+	+	−

[a] Length estimate in nucleotide base pairs.

[b] Polymorphic segments of DNA (replicated at least twice for verification); − indicates segment not amplified by single primer reaction; + indicates segment amplified by single primer reaction.

[c] Segment OPE-07 × 1000 was present in all eight single-basidiospore hyphal cultures. Between single drops of pycniospores, it was polymorphic.

DISCUSSION

In this study, polymorphic segments of genomic DNA were detected among hyphal cultures and among drops of pycniospores derived from a single-urediniospore culture of *C. q. fusiforme* using PCR with single, arbitrarily chosen primers. This study is the first to report on genetic variation within the formae speciales of *C. quercuum*.

To confirm that segments were amplified from genomic DNA, genomic DNA was omitted from control reactions. The pattern for every control reaction was erratic and not reproducible, or no amplification products were evident. Control-reaction products never affected amplification of polymorphic segments when genomic DNA was included in the reaction mixture. These results match results reported by other researchers (3,19,22).

The RAPD markers produced were repeatable and provided an effective method for preliminary identification of single-basidiospore hyphal cultures and single drops of pycniospores. Ten single-basidiospore hyphal cultures obtained from a single telial column derived from a single-urediniospore infection were distinguished. Tests detected differences between single drops of pycniospores collected from the same fusiform rust gall and between galls.

Given the conditions under which the pine seedlings were inoculated with basidiospores of *C. q. fusiforme*, it is probable that more than one basidiospore infected a seedling. The polymorphisms observed between amplified segments of genomic DNA from single drops of pycniospores within the same gall suggest that multiple genotypes of *C. q. fusiforme* must have been present in three of the four galls examined. The possible presence of more than one genotype of *C. q. fusiforme* within and between galls reinforces a belief that traditional experiments on the genetics of host-pathogen interactions between *Pinus* and *C. q. fusiforme* cannot be carried out using current methods of inoculation (10).

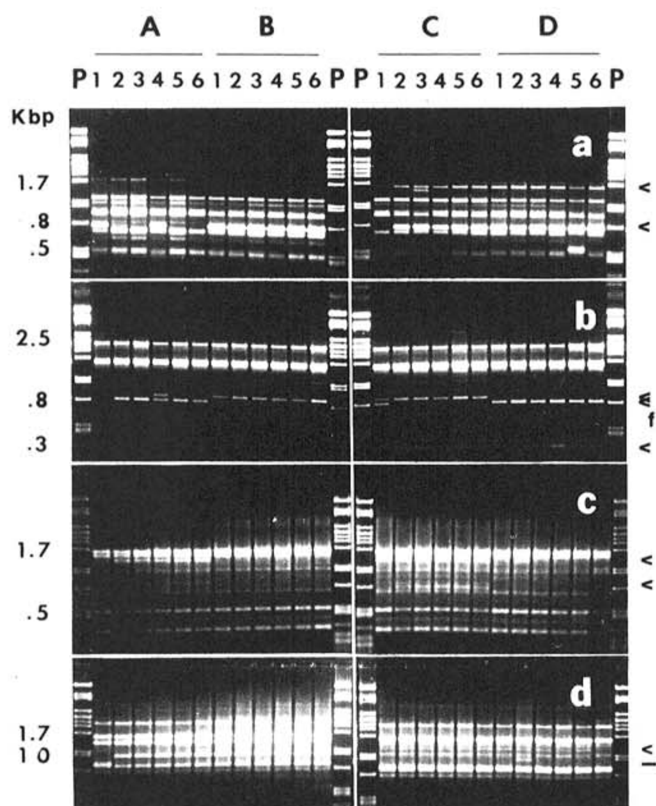

Fig. 2. Amplification of segments of genomic DNA from 24 separate, single drops of pycniospores of *Cronartium quercuum* f. sp. *fusiforme* collected from four fusiform rust galls (A, B, C, and D; lanes 1–6 show single drops of pycniospores within each gall). **a, b, c, and d,** show amplified segments for primers OPA-11, OPB-10, OPB-18, and OPE-07, respectively. < marks amplified segments polymorphic between the single drops of pycniospores; — is a segment polymorphic between single drops of pycniospores but monomorphic for the single-basidiospore hyphal cultures; and f identifies the position of a segment, OPB-10 × 0625, that is weakly amplified and too faint for reproduction in high-contrast photocopying. Drops can be distinguished by different patterns of polymorphisms. Amplified segments were resolved by electrophoresis through 2% agarose gels and were visualized by ethidium bromide staining. Size markers (P) are from *PstI* (I. B. I., New Haven, CT) restriction digestion of bacteriophage lambda (New England Biolabs, Beverly, MA).

Assaying these and additional polymorphic markers in this population of single-basidiospore hyphal cultures and single drops of pycniospores may permit the construction of a genetic linkage map of the single urediniospore-derived culture, WLP-10-2.SS1. Because urediniospores are asexual products of mitosis, the single urediniospore-derived culture used in this study presumably represents a single diploid genotype. Basidiospores are thought to be haploid products of meiosis, and pycniospores, through hyphal tissues and pycnia, are thought to be an extension of basidiospores. Thus, a population of basidiospores on telia arising from infection on oak by urediniospores, haploid mycelium obtained from single germinating basidiospores, and pycniospores on pycnia produced on mycelium from single-basidiospore infections on pine would be sibling gametes, gametothalli, and spermatia, respectively. The pattern of RAPD markers for the eight single-basidiospore hyphal cultures obtained from a single telial column suggest the cultures may represent a segregating population (Table 1). Of the 32 reproducible polymorphic segments, 27 showed 1:1 marker segregation of $\alpha \geq 0.05$ by chi-square analysis with one degree of freedom. Apparent recombination among some of the RAPD markers in the sample supports the inference that basidiospores are products of meiosis.

Given repeatable, RAPD markers, some proportion of which may be inherited in a Mendelian fashion, a detailed genetic linkage map could be produced for *C. q. fusiforme*. Single drops of pycniospores from 50 to 100 galls should provide a mapping population for WLP-10-2.SS1. Alternatively, additional single-basidiospore hyphal cultures obtained from the culture could be used. Such a genetic map would be invaluable for studying selection, identification, function, and organization of the *C. q. fusiforme* genome.

TABLE 2. Frequencies and distribution of random amplified polymorphic DNA (RAPD) phenotypes detected among 24 single drops of pycniospores based on amplification of 10 polymorphic segments of genomic DNA using polymerase chain reaction (PCR).[a]

Composite RAPD phenotype[c]	Composite phenotype detection: Gall[b] A	B	C	D	Total
−+−−++−−+−	1[d]	0	0	0	1
−−−++++−++	4	0	0	0	4
−++++++−++	1	0	0	0	1
−++−−−−−++	0	6	0	0	6
−−+++++−−−	0	0	1	0	1
+++−−++−−−	0	0	5	0	5
++−+++−−−−	0	0	0	1	1
++−++++−−+	0	0	0	5	5
Total	6	6	6	6	24

[a] Pycniospores were collected on fusiform rust galls resulting from inoculation of 9-wk-old loblolly pine seedlings with basidiospores derived from a single-urediniospore culture of *Cronartium quercuum* f. sp. *fusiforme*

[b] At least six single drops of pycniospores were collected from galls on each of four loblolly pine seedlings. Collected drops were distributed uniformly, no less than 1 cm apart, around the galls.

[c] Patterns of polymorphic segments of DNA amplified with single primers. Segments are OPA-11 × 1600, OPA-11 × 0800, OPB-10 × 0850, OPB-10 × 0805, OPB-10 × 0625, OPB-10 × 0340, OPB-18 × 1500, OPB-18 × 0900, OPE-07 × 1400, and OPE-07 × 1000. Polymorphic segments of DNA (replicated at least twice for verification): − indicates segment not amplified by primer; + indicates segment amplified.

[d] Number of times composite phenotype detected.

Genomic DNA markers can be immediately utilized in several study areas besides genetic mapping. A survey of *C. q. fusiforme* for range-wide genetic variation is being conducted using PCR with single primers. RAPD markers may be useful for characterization of within-stand variation. In studies on epidemiology, origins of infections might be identified, and relatedness between old and new infections could be compared. The RAPD markers should have useful applications in addressing questions on the basic biology of *C. q. fusiforme*, such as resolving the specifics on sexual reproduction and ploidy of spores in various stages of the life cycle. Taxonomic studies between *Cronartium* spp. and among formae speciales of *C. quercuum* could be facilitated using RAPD markers. Successful application of RAPD markers in studies of host-pathogen relationships in fusiform rust disease, however, remains dependent on an agressive classical genetics approach for both host and pathogen and on controlling variability in the pathogen using new breeding and inoculation techniques.

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