Interspecific and Intraspecific Differentiation Within the Genus *Cronartium* by Isozyme and Protein Pattern Analysis

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ABSTRACT

Powers, H. R., Jr., Lin, D., and Hubbes, M. 1989. Interspecific and intraspecific differentiation within the genus *Cronartium* by isozyme and protein pattern analysis. Plant Disease 73:691-694.

Isozyme analyses were made on aeciospore collections from several species of the genus *Cronartium* and from several formae speciales of *C. quercuum*. Aeciospores from closely related *Endocronartium harknessii* also were tested. Esterase isozyme patterns provided the best differentiation. Patterns for *Cronartium* spp. differed markedly from those for *E. harknessii*. *C. q.* f. sp. *banksianae*, *C. q.* f. sp. *virginianae*, *C. q.* f. sp. *echinatae*, and *C. q.* f. sp. *fusiforme* were easily distinguished. The tests did not detect variation within formae speciales.

The genus *Cronartium* includes species that cause some of the most damaging diseases of pines worldwide. *C. quercuum* (Berk.) Miyabe ex Shirai (eastern gall rust), which occurs throughout much of the northern hemisphere, includes closely related formae speciales that cause diseases on several pines in the eastern and southern United States. Fusiform rust of southern pines, caused by *C. q.* f. sp. *fusiforme* (=*C. fusiforme* Cumm.), is by far the most serious of these diseases. Eastern gall rust is now separated into three formae speciales on the basis of pine host species. Questions remain about the relationship between the causal organisms of fusiform rust and eastern gall rust, however. These fungi have similar life cycles, alternate hosts (*Quercus* spp.), and morphological characteristics. They differ in pine host preferences, and galls caused by fusiform rust are usually spindle-shaped (fusoid), whereas those caused by eastern gall rust on other pines are usually globose and possess bark collars at each end (10). Neither of these characteristics is entirely dependable, however. The fusiform rust fungus often produces irregularly shaped galls that may be globose and sometimes contain bark collars. Also, the fungus causing the eastern gall rust can produce galls that are irregular in shape.

Accepted for publication 5 March 1989.

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Inoculation studies using aeciospores collected from the various pine species have indicated consistent differences that could be termed "races" or "pathotypes" (11,13). For example, there were significant interactions between the responses obtained using aeciospores from jack pine (*Pinus banksianae* Lamb.) and Virginia pine (*P. virginianae* Mill.). The isolates from each species caused a significantly higher proportion of galls on their respective host (13). Other means of differentiation have included serological responses of fusiform rust on slash (*P. elliottii* Engelm. var. *elliottii*) and loblolly pine (*P. taeda* L.) and eastern gall rust on Virginia pine (9). Pathogenic interactions between these two rusts have been reported on various species of *Quercus* (5,7,8), as has a statistically significant aeciospore size difference (6). Because of the similarities in morphology, Burdsall and Snow (4) established formae speciales for the rusts on the various pine hosts. These included *C. q.* f. sp. *banksianae* on jack pine, *C. q.* f. sp. *virginianae* on Virginia pine, *C. q.* f. sp. *echinatae* on shortleaf pine, and *C. q.* f. sp. *fusiforme* on loblolly and slash pine.

Fusiform rust is a limiting factor in the production of loblolly and slash pines in much of the southern United States (14). As a result, there is much interest in development of rust-resistant pines and possible evolution of new strains of the fungus. New methods are urgently needed to identify pathotypes or virulent isolates of the fusiform rust fungus. Although no pathotypes of a pine tree rust have yet been identified by isozyme analysis, different geographic sources of *Endocronartium harknessii* (J. P. Moore) Y. Hirate., which causes pine-to-pine rust on jack pine, have been distinguished by this technique (18). Isozyme analysis has been used in several instances to differentiate species of plant pathogens and to identify races or strains of rusts on agronomic crops. For example, several species of *Peronosclerospora* were differentiated and their phylogenetic relationships were clarified by isozyme analysis (2). Isozyme analysis also made possible the evaluation of variation among numerous isolates of wheat stem rust caused by *Puccinia graminis* (Pers.) f. sp. *tritici* (3).

The objective of this study was to determine if it was possible to distinguish

between several species of the genus *Cronartium* by isozyme and protein pattern analysis of aeciospores. Further, we wanted to see if the four formae speciales within *C. quercuum* could be identified by these techniques.

MATERIALS AND METHODS

Aeciospores of the various species and formae speciales were collected from pine hosts and were processed and stored according to the procedures outlined by Roncadori and Matthews (15). Briefly, we collected the spores from fruiting rust galls, sifted the aeciospores through 150-μm mesh screens, and placed them into a desiccator containing $CaCl_2$. The aeciospores were kept in a desiccator at approximately 4 C for 2–3 wk until they became powdery in texture. Then 300 mg of aeciospores were placed into 5 ml freeze-drying ampoules and were dried for 2 hr at approximately 15–20μ of Hg.

Separate tests were made during 1984–1987. Each year a different group of aeciospores was collected, depending on the availability of fruiting rust galls. There were, however, several similar spore collections in each of the tests that could be used for comparisons. With two exceptions, each species and forma specialis was represented by collections from at least two geographic areas. Only one collection of the very rare *C. appalachianum* Hepting and only one isolate of *E. harknessii* were used. Table 1 lists the collections of each species and forma specialis and the type, host species, and state of origin for each.

For isozyme and protein analysis, 50 mg of aeciospores were ground for 30 sec in 0.5 ml Tris-HCl buffer solution (pH 6.8) with a Brinkmann homogenizer. Insoluble particles were sedimented on a high-speed microcentrifuge for 15 min at 15,000 *g*. The supernatants were collected, and the protein content was measured by the Bio-Rad protein assay method. The concentration of each sample was adjusted to 60 μg of protein/50 μl of buffer. Glycerol (20%, v/v) and a trace of bromophenol blue were added, and 50 μl of the sample was subjected to electrophoresis on 7.5% polyacrylamide slab gels at constant voltage of 200 V until the tracking dye had traveled to within 0.5 cm of the bottom of the gel. The discontinuous buffer system in the gel electrophoresis was prepared according to Laemmli (12). The gels were then stained for different enzymes (Table 2) according to the recipes of Shaw and Prasad (16) and Siciliano and Shaw (17). The R_f value of each respective band on schematic isozyme patterns was determined to allow precise comparisons among the various spore collections. The R_f value is the mobility of each isozyme band that traveled from the origin divided by the distance traveled by the front tracking dye.

For protein patterns analysis, a 7–15% gradient polyacrylamide gel was prepared with the discontinuous buffer system of Laemmli (12). The gel was stained overnight in 0.025% Coomassie Blue solution containing water (45%), acetic acid (10%), and methanol (45%). The gel was destained with 7% (v/v) acetic acid and 5% (v/v) methanol in water until the solution became clear. The optical density scan for protein patterns was obtained by scanning photographs of the gels with a Zeiss PMQ-3 spectrophotometer at 550 μm.

Table 1. Aeciospore isolates used in isozyme analysis, with type of collection, host species, and state of origin

Fungus	No. of collections	Type of collection	Host species	State of origin
Cronartium quercuum f. sp. *fusiforme*	42	Mass (− 9) Single gall (−33)	Loblolly (39) Slash (1) Pond (1) Longleaf (1)	Georgia (17) South Carolina (15) Louisiana (4) Texas (3) Maryland (2) Alabama (1)
C. q. f. sp. *virginianae*	7	Mass (− 3) Single gall (− 4)	Virginia (7)	South Carolina (5) North Carolina (2)
C. q. f. sp. *echinatae*	2	Mass (− 1) Single gall (− 1)	Shortleaf (2)	Mississippi (2)
C. q. f. sp. *banksianae*	2	Mass (− 2)	Jack (2)	Minnesota (1) Wisconsin (1)
C. comandrae	3	Mass (− 3)	Loblolly (3)	Tennessee (3)
C. appalachianum	1	Mass	Virginia (1)	Tennessee (1)
C. ribicola	2	Mass (-2)	Eastern white (2)	North Carolina (2)
Endocronartium harknessii	1	Mass	Jack (1)	Minnesota (1)

Table 2. Enzymes tested for aeciospores

Hydrolases, lyases, and transferases	Enzyme commission number	Reaction	Differentiation
Acid phosphatase (AP)	3.1.3.2.	−	...
Alkaline phophatase (AKP)	3.1.3.1.	−	...
Esterase (EST)	3.1.1.1.	+	+
Leucine aminopeptidase (LAP)	3.4.11.1.	−	...
Phosphoglucomutase (PGM)	2.7.5.1.	−	...
Dehydrogenases			
Alcohol dehydrogenase (ADH)	1.1.1.1.	−	...
Glucose-6-phosphate dehydrogenase (G6PDH)	1.1.1.49.	+	−
α-Glycerophosphate dehydrogenase (α-GPDH)	1.1.1.8.	−	...
Isocitrate dehydrogenase (IDH)	1.1.1.42.	−	...
Lactate dehydrogenase (LDH)	1.1.1.27.	−	...
Malate dehydrogenase (MDH)	1.1.1.37.	+	−
Others			
Glucose phosphate isomerase (GPI)	5.3.1.9.	+	+
Glutamate oxaloacetate transaminase (GOT)	2.6.1.1.	−	...
Hexokinase (HEX)	2.7.1.1.	−	...
Malic enzyme (ME)	1.1.1.40.	+	−
Peroxidase (PER)	1.11.1.7.	−	...

RESULTS

Of 16 enzyme systems tested, five gave positive reactions (Table 2). Two enzymes, esterase and glucose phosphate isomerase, differentiated among the species and formae speciales of the genus *Cronartium*. In general, esterase consistently provided the best differentiation. In a test of four species of *Cronartium* and *E. harknessii*, very sharp differences were observed (Fig. 1). In this comparison, *C. appalachianum* could not be characterized due to the weak reaction to the staining solution. However, the general protein patterns of this species distinguished it very clearly from *C. comandrae* Peck and *E. harknessii* (Fig. 2). More precise differentiation of these species was obtained by the spectra of their protein patterns (Fig. 3). *C. comandrae* has specific protein bands at R_f 0.12, 0.57, 0.59, 0.68, and 0.71, whereas *C. appalachianum* has specific bands at R_f 0.44, 0.67, and 0.72 and *E. harknessii* at R_f 0.52, 0.54, 0.64, 0.66, and 0.69 (Fig. 3). Some bands are shared by two or all three of the species.

C. q. f. sp. *banksianae*, *C. q.* f. sp.

virginianae, *C. q.* f. sp. *echinatae*, and *C. q.* f. sp. *fusiforme* were easily distinguished by their esterase isozyme patterns (Fig. 4). Collections of the same formae speciales differed in intensity of banding, but not in position of the bands. This variation in intensity, which was observed in several tests of different collections of *C. q.* f. sp. *fusiforme*, does not necessarily indicate variation among different collections of a single forma specialis. The R_f values of the esterase isoyzme patterns of the spore collections of several species and formae speciales of *Cronartium* and *E. harknessii* are shown in Figure 5. For comparisons of the formae speciales of *C. quercuum*, *C. q.* f. sp. *banksianae* served as the standard. The results show *C. q.* f. sp. *fusiforme* is different from *C. q.* f. sp. *banksianae* at R_f 0.43, 0.48, and 0.54; *C. q.* f. sp. *echinatae* is different at R_f 0.42 and 0.46; and *C. q.* f. sp. *virginianae* does not possess a band at R_f 0.55. *C. comandrae* has unique esterase bands at R_f 0.62–0.72, and *C. ribicola* J. C. Fischer ex Habh. has such bands at R_f 0.64–0.69. All *Cronartium* species had a protein band at R_f 0.59, whereas this was not observed in *E. harknessii*. Results of preliminary efforts to distinguish between the collections of *C. quercuum* f. sp. *fusiforme* from galls of differing shape have been inconclusive.

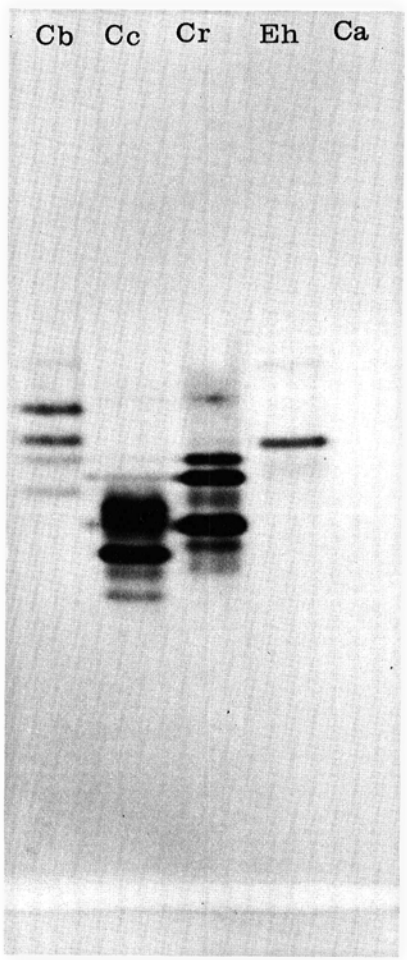

Fig. 1. Esterase patterns of aeciospores from *Cronartium quercuum* f. sp. *banksianae* (Cb), *C. comandrae* (Cc), *C. ribicola* (Cr), *Endocronartium harknessii* (Eh), and *C. appalachianum* (Ca).

DISCUSSION

Most species of the genus *Cronartium*, and even the four formae speciales within the species *C. quercuum*, can be distinguished by gall morphology, host preference, or, in the case of comandra rust, by the shape of the aeciospores. The closely related pine-to-pine rust, *E. harknessii*, which attacks jack pine, can be distinguished from the strain of *C. quercuum* that occurs on jack pine either by inoculation tests or the characteristics of germinating aeciospores (1). Our study was initiated to determine whether isozyme and protein analysis could distinguish among *E. harknessii* and several different species of *Cronartium*. Another goal was to see if identifications could be made at the formae speciales level within the species *C. quercuum*.

Our comparisons involved a wide range of species and isolates of the genus *Cronartium*. As expected, differences were greatest among the different species of *Cronartium* and between *E. harknessii* and all other species. The *E. harknessii* collection was by far the most distinctive in that it lacked a zymogram band that was present in all collections of *Cronartium* species. Although formae speciales of *C. quercuum* were separable, they did share a number of common bands. Our ability to separate the different formae speciales of *C. quercuum* confirms the classification work by Burdsall and Snow (4). Apparently, there are clear physiological differences among the four formae speciales that were established based on host preferences.

Our long-term objective is to distinguish among strains or races of tree rust fungi with differing levels of virulence. Because new rust races on agronomic crops have been identified by these techniques, such differentiation also may be possible with tree rusts. If new races of the rusts could be identified by isozyme analysis, we could eliminate time-consuming inoculation tests and the inherent variability introduced by going through the sexual stage of the organism

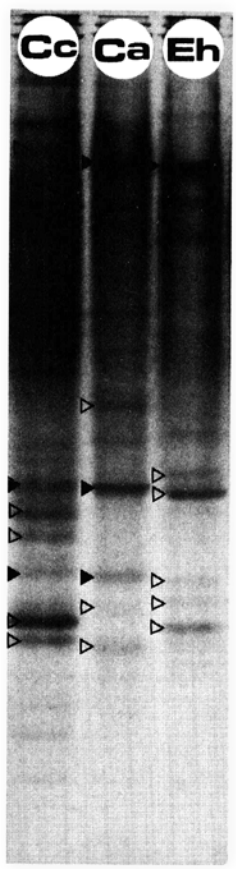

Fig. 2. Protein patterns of aeciospores from *Cronartium comandrae* (Cc), *C. appalachianum* (Ca), and *Endocronartium harknessii* (Eh).

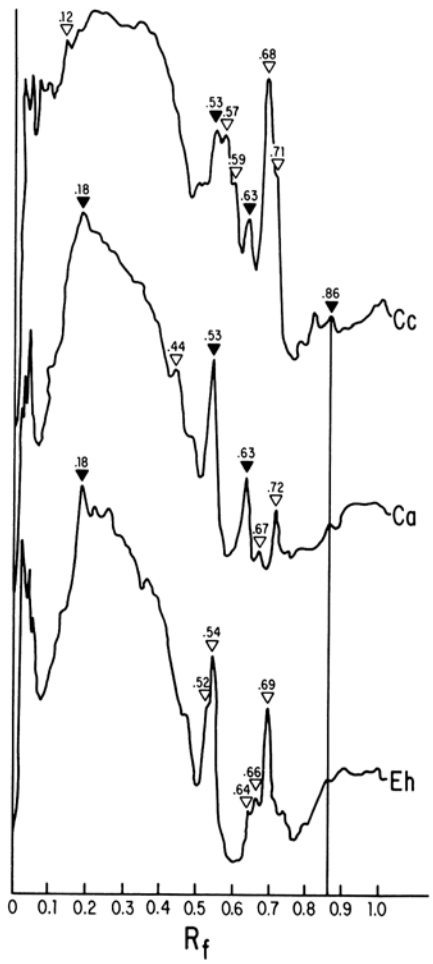

Fig. 3. Spectra of protein patterns of aeciospores from *Cronartium comandrae* (Cc), *C. appalachianum* (Ca), and *Endocronartium harknessii* (Eh). Open triangles show protein bands for specific R_f values distinct for that species. (R_f value is the mobility of each isozyme band that traveled from the origin divided by the distance traveled by the front tracking dye.) Closed triangles indicate bands shared by at least one other species.

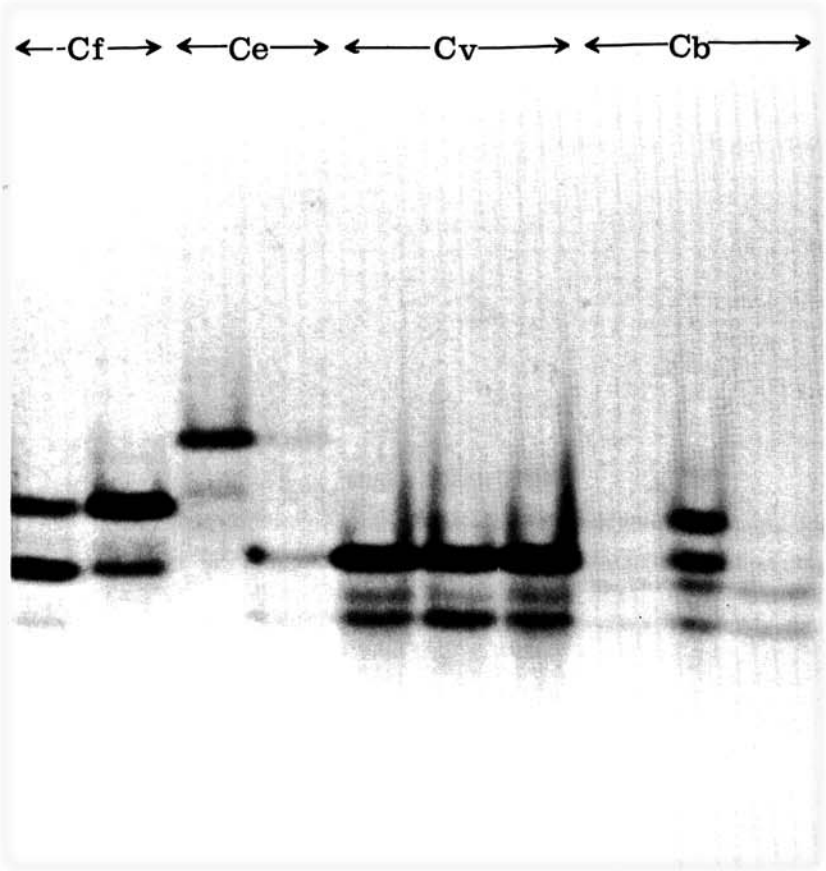

Fig. 4. Esterase patterns of *Cronartium quercuum* f. sp. *fusiforme* (Cf), *C. q.* f. sp. *echinatae* (Ce), *C. q.* f. sp. *virginianae* (Cv), and *C. q.* f. sp. *banksianae* (Cb).

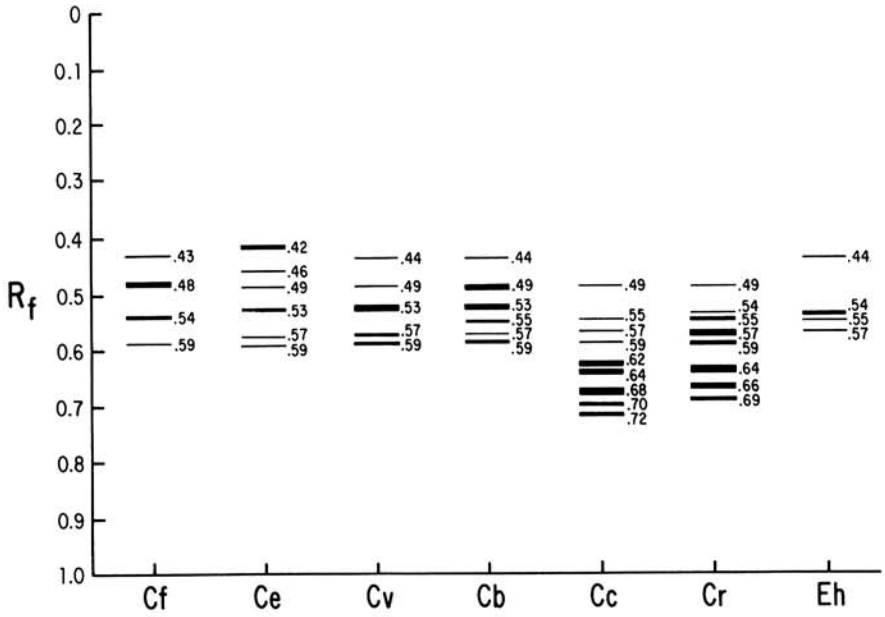

Fig. 5. R_f values of esterase patterns of *Cronartium quercuum* f. sp. *fusiforme* (Cf), *C. q.* f. sp. *echinatae* (Ce), *C. q.* f. sp. *virginianae* (Cv), *C. q.* f. sp. *banksianae* (Cb), *C. comandrae* (Cc), *C. ribicola* (Cr), and *Endocronartium harknessii* (Eh). (R_f value is the mobility of each isozyme band that traveled from the origin divided by the distance traveled by the front tracking dye.)

on the alternate host in order to produce the basidiospores needed for inoculation tests.

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