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Differentiation among *Cronartium* Species from Northeast China by Isozyme Analysis

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SUMMARY: Isozyme analysis was conducted on aeciospores of three pine-stem rust species of the genus *Cronartium* found in the northeastern China. Aeciospores of *Coleosporium pulsatillae*, a pine-needle rust, were also tested. Of the seven enzymes tested, esterase (EST) isozyme patterns provided the best differentiation, and distinctly separated *C. ribicola*, *C. flaccidum*, and *C. quercuum* from one another. The EST isozyme patterns for *Coleosporium pulsatillae* differed remarkably from all three *Cronatium* species as expected. Two EST electromorphs were found in five isolates of *C. flaccidum*, suggesting that the genetical variation in the sampled population of this species is big. The EST isozyme patterns of *C. ribicola* from northeastern China are consistently in good agreement with those of *C. ribicola* from the United States previously reported, possibly implicating that the selection pressure from the geographical separation has been minimal on the differentiation of this rust during evolution.

Key Words: *Cronartium*, taxonomy, isozyme analysis, genetical variation.

INTRODUCTION

The genus *Cronartium* includes several species which induce rust diseases on the stems of pines worldwide. In northeastern China, there are three *Cronartium* species, namely *C. ribicola*, *C. flaccidum*, and *C. quercuum*. *C. ribicola occurs* on Korean pine (*Pinus koraiensis*) which is a five-needle conifer. *C. flaccidum* infects two-needle pines including Mongolian pine (*Pinus sylvestris* var. *mongolica*), Xinkai-lake pine (*Pinus takahashii*), and Chinese pine (*Pinus tabulaeformis*) in this area. Another pine-stem rust is *C. quercuum* which attacks mongolian pine and Xinkai-lake pine to induce globose galls on stems and branches.

The rust diseases caused by these pathogens are a limiting factor in the production of pines in northeastern China. Identifying pathogens and characterizing genetical variations of them are important components in the study of disease systems. Since there are few apparent morphological differences among these three *Cronartium* species, studies were conducted to distinguish them on the basis of electron microscopical morphology of aeciospores (2, 3, 7).

Isozyme analysis offers one method of identifying species or races of a pathogen as well as detecting variations in pathogens based on molecular genetics. This technique has been used recently to study the genetical variation in *Peridermium harknessii* (8) and to differentiate the species and formae speciales of *Cronartium* (5) occurring in the United States. Because to date, there is no published information on the identification of *Cronartium* rusts occurring in China using isozyme data, we conducted the present study with a goal to determine if it was possible to distinguish among the three *Cronartium* species found in the northeastern China by isozyme analysis of aeciospores.

MATERIALS AND METHODS

Separate aeciospore-collections and isozyme analysis tests were conducted during 1992-1994. Each year a different group of aeciospores was collected, depending on the availability of spores (Table 1). The aeciospores of *C. ribicola* and *C. flaccidum* were collected from single trees, so that each collection is considered as one isolate of that species. The spores of *C. quercuum* were mass samples due to scarcity of the fruiting in the collection year. Aeciospores from a pine-needle rust, *Coleosporium pulsatillae*, were also collected from individual trees for isozyme analysis. Each sample of spores was passed through a 150-μm mesh screen, placed in a desiccator containing $CaCl_2$, and kept at approximately -15 C for 30-70 days before the homogenization.

For enzyme extraction, 30 mg of aeciospores of each

sample were hand-ground using a pestle for 1 min in a mortar containing about 20 mg 400# carborundum and 0.3 ml Tris-HCl extraction buffer solution (pH 7.0). Other extraction additives included 10 mM dithiothretol (DTT) and 10 mM vitamin C. After the grinding, about 30 % of the spores was disrupted. All samples were kept on ice during preparation. Insoluble particles were sedimented on a high-speed microcentrifuge for 7 min at 6000g. The supernatants were used immediately or with a storage of 3-5 days at -15 C.

Electrophoresis tests were conducted using procedures described by Shiraishi (6). The discontinuous buffer system in the gel electrophoresis was used. 10 µl of the sample was subjected to electrophoresis on 7.5 % polyacrylamide slab gels (1.5 mm in thickness) until the tracking dye had traveled to within 1 cm of the bottom of the gel. The gels were stained for different enzymes (Table 2) according to the recipes of Murphy et al. (4). For *C. quercuum*, the staining was conducted only with esterase due to the scarcity of the quantity of aeciospores collected. The Rf value of each respective band on schematic isozyme patterns was determined to allow precise comparisons among the various spore samples. The Rf value is the mobility of each isozyme band that traveled from the origin divided by the distance traveled by the front tracking dye. Three different runs of the electrophoresis tests were made, and each provided identical results.

RESULTS

Of the seven enzymes tested, six gave positive staining reaction in at least one of the tested species (Table 3). However, only the enzyme EST produced distinctive isozyme patterns for all three *Cronartium* species, and also differentiated best among them. The isolates of *C. ribicola* collected in 1992 and those in 1994 differed in EST isozyme band number from each other due to the variation of staining intensity (Fig. 1). However, they have common bands at same Rf value (0.55 and 0.64) to indicate the identity of their isozyme patterns.

The Rf values of EST isozyme bands for the four rust species tested are shown in Fig. 2, where the band pattern for *C. ribicola* are based on the 1992 test (Fig. 1A) which presented more bands than the 1994 test (Fig. 1B). As

Table 1. Host species, number of samples and origins of aeciospores used in isozyme analysis.

Rust fungus	Host	No. of samples*	Origin * *
Cronartium ribicola	*Pinus koraiensis*	6	Caohekou, LN
		3	Xinbin, LN
		5	Caohekou, LN
C. flaccidum	*P. sylvestris* var.*mongolica*	5	Jagedaqi, HLJ
C. quercuum	*P. takahashii*	Mass	Mishan, HLJ
Coleosporium pulsatillae	*P. tabulaeformis*	3	Zhanggutai, LN

* A sample refers to all spores collected from an individual tree.
* * LN=Liaoning province; HLJ=Heilongjiang province.

Table 2. Enzymes tested for aeciospores.

Enzyme	Abbreviation	Enzyme commission number
Acid phosphatase	ACP	3.1.3.2.
Esterase	EST	3.1.1.1.
Glucose-6-phosphate dehydrogenase	G6P	1.1.1.49
Glutamate oxaloacetate transaminase	GOT	2.6.1.1.
Malate dehydrogenase	MDH	1.1.1.37
Malic enzyme	ME	1.1.1.40
Peroxidase	PER	1.11.1.7.

Table 3. Staining reactions of the enzymes for the four species of rust fungi.

	ACP	EST	G6P	GOT	MDH	ME	PER
Cronartium ribicola	*	+	*	–	+	–	–
C. flaccidum	*	+	*	–	+	+	+
C. quercuum	/	+	/	/	/	/	/
Coleosporium pulsatillae	–	+	*	–	+	+	–
Differentiation of pattern	no	yes	no	no	no	yes	no

+: Band distinguishable in at least one of the tested isolates.
–: No band observed; *: Smeared pattern; /: Staining test not conducted.

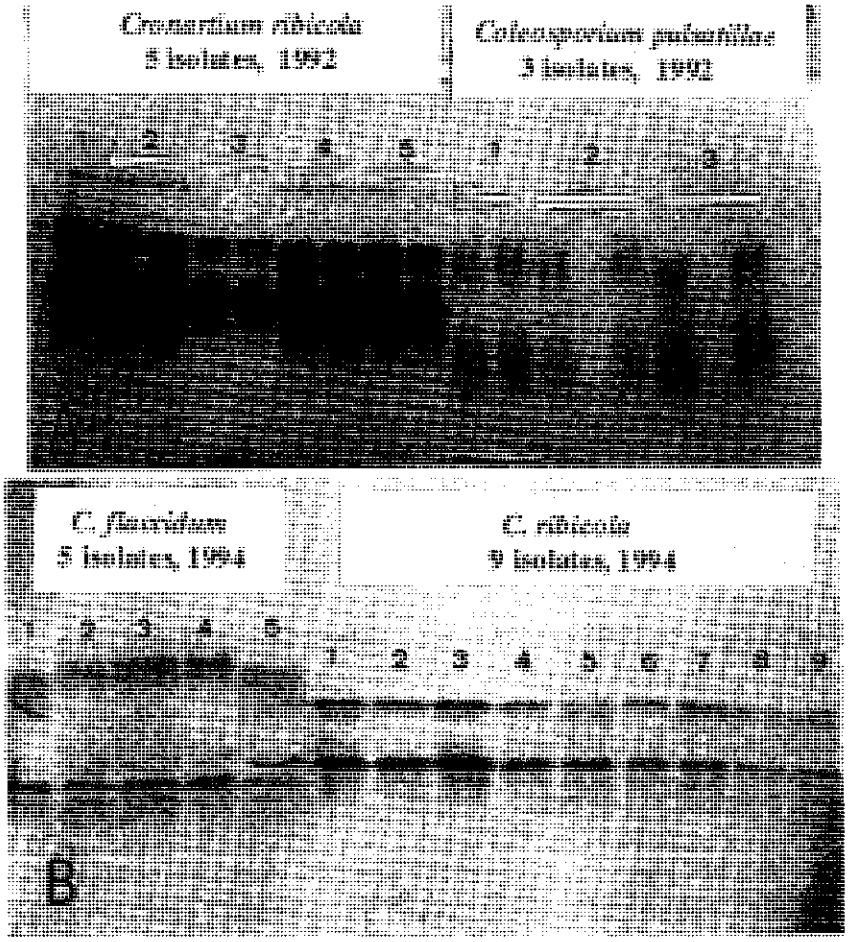

Fig. 1. Esterase patterns of aeciospores from different isolates of pine rust fungi. A: *Cronartium ribicola* (5 isolates) and *Coleosporium pulsatillae* (3 isolates) tested in 1992; B: *C. flaccidum* (5 isolates) and *C. ribicola* (9 isolates) tested in 1994. Note that the *C. ribicola* isolates of No. 4-8 tested in 1994 (Fig 1B) were collected from the same individual trees as were the five *C. ribicola* isolates (No. 1-5) tested in 1992.

shown in Fig. 2, *C. ribicola* shows five bands at Rf ranging from 0.5 to 0.7. *C. flaccidum*, which displayed two electromorphs, has one band at Rf 0.47 (or at 0.51 in another electromorph) and the other at Rf 0.67. *C. quercuum* possesses four bands at Rf ranging from 0.38 to 0.62. The pine-needle rust, *Coleosporium pulsatillae*, which showed only two faint EST bands at Rf 0.56 and Rf 0.73, can be easily distinguished by the banding patterns from the three

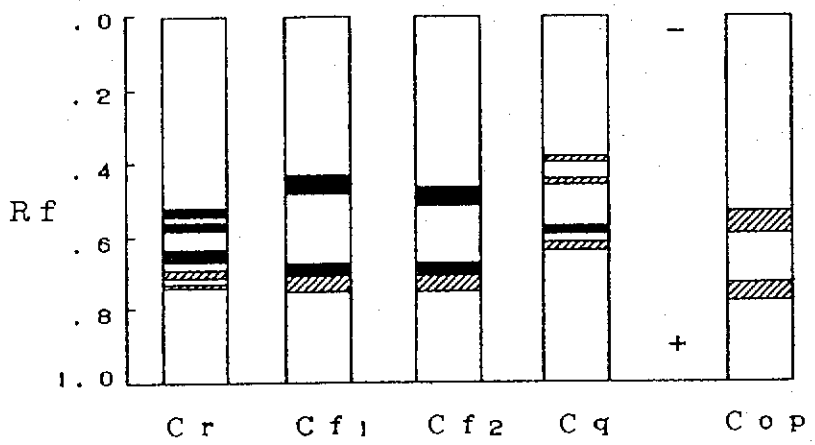

Fig. 2. Rf values of esterase patterns of aeciospores from *Cronartium ribicola* (Cr), *C. flaccidum* (Cf), *C. quercuum* (Cq), and *Coleosporium pulsatillae* (Cop). Rf is the mobility of each isozyme band expressed as a percentage equal to the migrated distance divided by the distance the front tracking dye migrated.

Cronartium species.

DISCUSSION

Based on several electron microscopic observations of the surface morphology of aeciospores (2, 3, 7), Liu et al. concluded that *C. ribicola* and *C. flaccidum* found in China could be reasonably treated as two separate species. Our study for the first time distinguished *C. ribicola* and *C. flaccidum* on the basis of isozyme data, thus providing molecular genetical evidence which supports the conclusion of Liu et al.

Two EST electromorphs (Cf1 and Cf2) occurring in five isolates of *C. flaccidum* (Figs. 1 and 2) possibly suggests that the genetical variation in the sampled population of this rust is big. In contrast, the remarkable consistency of the EST patterns shown in nine isolates of *C. ribicola* seems to indicate that the genetical variation in *C. ribicola* population sampled is small.

By comparing EST isozyme patterns, Powers et al. (5) successfully separated the four American formae speciales of *C. quercuum* established on the basis of pine host species. In his study, however, the different formae speciales, though their EST isozyme patterns distinguishable from one another, did share a number of common bands. In general, the more common isozyme bands present, the closer the relatedness of the compared taxonomic units can be considered to be. Our study showed that among the EST isozyme patterns produced by the three *Cronartium* species we tested, there is no common bands at all. This result provided new evidence that the consideration of *C. ribicola*, *C. faccidum*, and *C. quercuum* as separate species is fairly reasonable.

The *C. ribicola* isolates tested in our works were collected on Korean pine from the northeastern China, whereas those in Powers' study (5) were obtained on white pine (*Pinus strobus*) from the eastern United States. However, the EST isozyme patterns of *C. ribicola* shown in our repeated tests (Fig. 1) are consistently in good agreement with those reported by Powers et al. This consistency of the isozyme patterns implies that the geographical separation and/or the host differences have not exerted much influence on the genetical differentiation of this five-needle-pine stem rust during evolution.

Our study was initiated to distinguish among the three *Cronartium* species occurring in northeastern China through isozyme analysis. Our long-term objective is to identify new races or strains of pine-stem rusts which have different host specificity and to detect the relationship between host preference and the genetical variation. If new races of a rust could be detected by isozyme analysis, we could eliminate time-consuming inoculation tests in the identification of rust fungi as well as in the study of host-rust pathogen interrelations.

Powers et al. detected the variation among formae speciales of *C. quercuum* using isozyme data as stated above. Whether such variation exists in *C. quercuum* or other pine-stem rusts occurring in China is an interesting question to be answered. Comparisons among *Cronartium* rusts found not only in China but also in the other parts of the world on the basis of molecular genetical ground may lead to better understanding of the taxonomy, the origin, the dispersion routes, or the evolution history of the *Cronartium* rusts. We have initiated a molecular systematical study on the *Cronartium* rusts found in China, and if possible also including those occurring abroad, using

isozyme as well as DNA polymorphism (for example, RAPD) analysis techniques (1, 9). Our work is just under way.

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