Techniques

# Isozyme Characterization of *Peridermium harknessii* and *Cronartium quercuum* f. sp. *banksianae* with Starch Gel Electrophoresis

G. A. Tuskan and J. A. Walla

Assistant professor, Department of Horticulture and Forestry, and research associate, Department of Plant Pathology, respectively, North Dakota State University, Fargo 58105.
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## ABSTRACT

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Starch gel electrophoresis techniques were examined and modified for utilization in characterizing variability in *Peridermium harknessii* and *Cronartium quercuum* f. sp. *banksianae*. Alternative spore preparation techniques, extraction buffer components, and enzyme staining systems are presented. Eleven of the 33 examined enzymes produced useful isozyme data. Allozyme frequencies between *P. harknessii* and *C. q. banksianae* were significantly different for catalase and phosphoglucomutase. Interpretation of the data indicates that the sampled *P. harknessii* population is heterogeneous, homozygous, and that the *C. q. banksianae* population is heterogeneous and heterozygous.

*Additional keywords*: Eastern gall rust, *Endocronartium harknessii*, western gall rust.

*Peridermium harknessii* Moore (*Endocronartium harknessii* (Moore) Hiratsuka), the causal organism of western gall rust, occurs throughout western and northern North America and infects more than 20 native and exotic hard pine species. Damage includes reduced growth rates, death of lateral branches, loss of the terminal leader, and reduced economic value of infected trees. Several tree improvement programs have been initiated to identify and develop resistant host selections. Programs are under way for lodgepole pine (*Pinus contorta* Dougl. ex Loud.) (3), jack pine (*Pinus banksianae* Lamb.) (11), Monterey pine (*Pinus radiata* D. Don.) (19), and ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) (22). In most cases, resistance evaluations are based on field performance of host trees against endemic *P. harknessii* populations. Little is known about genetic variability in virulence or host preference in the pathogen.

*Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *banksianae*, the causal organism of eastern gall rust, has a similar host range, causes similar damage, and occurs sympatrically with *P. harknessii* in the northern portion of its distribution. Despite morphological similarities, the life cycles of *C. q. banksianae* and *P. harknessii* are quite different. *C. q. banksianae* is a heteroecious, macrocyclic rust, whereas *P. harknessii* is an autoecious, microcyclic rust. Tree improvement programs have also been initiated to identify resistance to *C. q. banksianae* (11), and as with *P. harknessii*, little is known about genetic variability in the pathogen.

Electrophoretic techniques have been used to investigate genetic variability in other organisms (14,17,21). Differences between *P. harknessii* and *C. q. banksianae* and, more importantly, differences among isolates within species may be detectable by using electrophoretic techniques. Identification and characterization of isozymes in an organism are necessary before genetic variability can be quantified electrophoretically. The purpose of this investigation was to adapt and develop techniques necessary to characterize isozymes in *P. harknessii* and *C. q. banksianae*. Those techniques, as well as the resulting data from regional populations of *P. harknessii* and *C. q. banksianae*, are reported.

## MATERIALS AND METHODS

**Tissue collection and preparation.** In 1986 and 1987 spores were collected from 166 individual galls of *P. harknessii* from 11 sites throughout North Dakota and northwestern Minnesota (Fig. 1). Host species included ponderosa, jack, and Scots (*Pinus sylvestris* L.) pines. Spores were collected with a vacuum cyclone spore collector, passed through a 90-$\mu$m mesh sieve, placed in cryovials, desiccated under vacuum, and then stored at −60 C. Spores collected from individual galls were kept separate. Spores were also collected from 31 individual galls of *C. q. banksianae* on jack pine from two sites in northwestern Minnesota (Fig. 1). Identification of rust species was based on spore germination characters (1).

One day before electrophoresis, 20 mg of spore samples was removed from the freezer and placed into a 0.5-ml microcentrifuge tube containing 10 mg of #400 carborundum and 100 μl of 0.5 M sodium phosphate extraction buffer, pH 7.5. Other extraction additives, for example, 0.1% (w/v) dithiothreitol (DTT), 20 μl of X-77, 1% (w/v) bovine serum albumin (BSA), 1% (w/v) polyvinylpolypyrrolidone (MW = 40,000) (PVP-40), and 10 mM phenylmethanesulfonyl fluoride (PMSF) in acetone, were also tested as means of improving enzyme activity or isozyme resolution. Vials containing the spores, carborundum, and the extraction buffer were vortexed to suspend the normally hydrophobic spores. The suspension was frozen (−20 C) overnight. Frozen spore suspensions were thawed and mechanically macerated for 60 sec with a spinning poly(tetrafluoroethylene) grinding head manufactured to fit into the microcentrifuge tube. Approximately 55% of the spores was disrupted. The crude homogenate was immediately absorbed onto three 2.5 cm × 0.2 cm wicks cut from Whatman's no. 3 chromatographic paper. All samples were kept on ice during preparation.

**Electrophoresis and enzyme detection.** Three buffer systems, morpholine-citrate (8), histidine-citrate (9), and lithium-borate (20) were used to examine 33 enzymes for the tested spore samples. A list of tested enzymes, E.C. numbers and abbreviations, the corresponding buffer systems, and the references for enzyme staining formulas are presented in Table 1. Gels (18 × 15 × 2.2 cm) were prepared by using 10.5% (w/v) Sigma electrophoresis starch in 750 ml of each of three buffer composites and were used the same day. Wicks with homogenate from each sample were placed into one of 36 lanes at the origin of each gel. Spores from one of two large single gall collections from site 12 were used as a standard for all runs. Gels were electrophoresed for 20 min before the wicks were removed. Residual spore particles on the gel were removed with a moist brush. Current was reapplied until the dye front had migrated about 10 cm from the cathodal origin. Current was set at 50 mA for approximately 5 hr, 60 mA for approximately 5.5 hr, and 75 mA for approximately 6.5 hrs for the morpholine-citrate, histidine-citrate, and lithium-borate buffer systems, respectively. After electrophoresis, each gel was cut horizontally 11 times into about 2-mm-thick slices. Each slice was then placed in an enzyme staining tray that contained the substrate, dye, and cofactors required for each enzyme reaction and that had been prepared just before use.

Classification and comparisons of isozymes were based on the mobility of each zone of activity and the most common banding pattern within each zone of activity. For example, for enzymes that display multiple zones of activity, the zones were numbered sequentially from the anode to the cathode. Individual bands were numbered 1 for the most frequent, 2 for the next most frequent, and so on. The mobility of each band was expressed as a percentage equal to the migrated distance divided by the distance the buffer front migrated, that is, an Rf value.

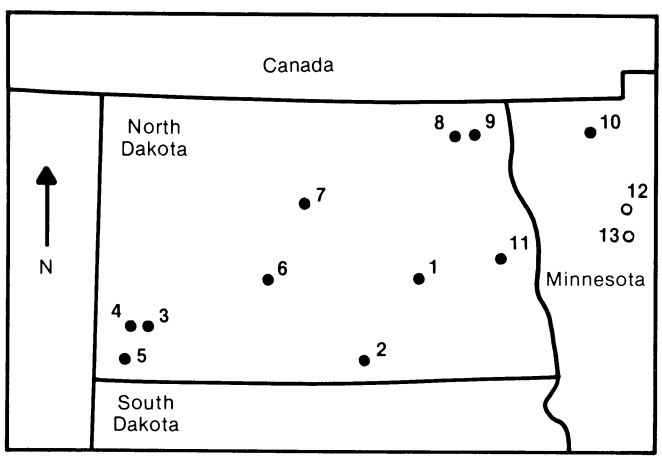


**Fig. 1.** The geographic distribution of *Peridermium harknessii* (•) and *Cronartium quercuum* f. sp. *banksianae* (o) isolates that were sampled for electrophoretic study.

## RESULTS AND DISCUSSION

**Modification of electrophoretic procedure.** The length of time spores were stored had no effect on enzyme expression, except for catalase, which had faint but still readable bands when stored longer than 1 yr. Similarly, no differences were noted in enzyme expression between spores stored in liquid nitrogen (−96 C) and those stored in an ultra cold freezer (−60 C). The use of gels prepared the day before electrophoresis resulted in fainter and more dispersed band patterns than gels prepared the same day. Freezing spore suspensions before maceration or adding carborundum improved ease of maceration without altering band mobility.

Extraction buffer additives were not useful. Addition of X-77 improved spore wetting, but caused a number of enzymes not to stain or to stain very faintly. The addition of PMSF as a protease inhibitor resulted in the loss of all enzyme activity. Incorporating PVP-40 to increase homogenate viscosity produced no appreciable improvement. The addition of DTT as an antioxidant and BSA to bind phenolics and free fatty acids resulted in no detectable change in band intensity. Subsequently, the above additives were not included in the extraction buffer.

**Enzyme characterization.** In *P. harknessii*, 20 of the 33 examined enzymes produced either no activity or faint, dispersed bands that could not be interpreted. Enzymes that were not present or in which activity was not detected included ADH, AAP, ALD, FDP, FUM, GLD, G3D, LAP, PPC, PPO, SDH, SUD, and XDH. The 50-ppm Amido Black stain for general protein also produced no visible staining. Enzymes with faint activity included ACO, ADK, GDH, HXK, IDH, MAE, and SKD. Modifying the preparation techniques or changing the buffer system did not

TABLE 1. Enzyme classification, identification of the tested buffer systems, and reference to enzyme staining recipes for use with *Peridermium harknessii* and *Cronartium quercuum* f. sp. *banksianae*

| Enzyme (abbreviation) | E.C. number | Buffer system[a] | Stain recipe reference[b] |
|---|---|---|---|
| Acid phosphatase (ACP) | 3.1.3.2 | M | 9,10,16 |
| Aconitase (ACO) | 4.2.1.3 | M | 10,16 |
| Adenylate kinase (ADK) | 2.7.4.3 | H | 9,16 |
| Alanine aminopeptidase (AAP) | 3.4.11.1 | L | 9,10 |
| Alcohol dehydrogenase (ADH) | 1.1.1.1 | L | 9,10,16 |
| Aldolase (ALD) | 4.1.2.13 | H,M | 9,10 |
| Alkaline phosphatase (ALP) | 3.1.3.1 | H | 10 |
| Catalase (CAT) | 1.11.1.6 | L,H | 9 |
| Diaphorase (DIA) | 1.6.4.3 | M,H | 9,16 |
| Esterase (EST) | 3.1.1.1 | H | 9,10,16 |
| Fructose 1-6 diphosphatase (FDP) | 3.1.3.11 | M | 16 |
| Fumerase (FUM) | 4.2.1.2 | H | 9,16 |
| Glucose-6-P-dehydrogenase (G6P) | 1.1.1.49 | M | 9,10,16 |
| Glutamate dehydrogenase (GDH) | 1.4.1.3 | L | 9,16 |
| Glutamic oxaloacetic transaminase (GOT) | 2.6.1.1 | L,H | 10,16 |
| Glyceraldehyde-3-P-dehydrogenase (G3D) | 1.2.1.12 | H | 9,16 |
| Glycerate dehydrogenase (GLD) | 1.1.1.29 | M,H | 9 |
| Hexokinase (HXK) | 2.7.1.1 | H | 9 |
| Isocitrate dehydrogenase (IDH) | 1.1.1.42 | M | 10,16 |
| Leucine aminopeptidase (LAP) | 3.4.11.1 | L | 9,10,16 |
| Malate dehydrogenase (MDH) | 1.1.1.37 | M | 10,16 |
| Malic enzyme (MAE) | 1.1.1.40 | M | 9,16 |
| Mannose phosphate isomerase (MPI) | 5.3.1.8 | M,L | 9,10,16 |
| Menadione reductase (MNR) | 1.6.99.2 | H,L | 9,10,16 |
| Phosphoenolpyruvate carbonylase (PPC) | 4.1.1.31 | L | 9 |
| Phosphoglucomutase (PGM) | 2.7.1.5 | H | 9,16 |
| 6-Phosphogluconate dehydrogenase (6PG) | 1.1.1.44 | M | 10,16 |
| Phosphoglucose isomerase (PGI) | 5.3.1.9 | L,M | 9,10 |
| Polyphenol oxidase (PPO) | 1.14.18.1 | L | 9 |
| Shikimate dehydrogenase (SKD) | 1.1.1.25 | M,H | 9,10 |
| Sorbitol dehydrogenase (SDH) | 1.1.1.14 | L,H | 9 |
| Succinate dehydrogenase (SUD) | 1.3.99.1 | H | 9 |
| Xanthine dehydrogenase (XDH) | 1.2.1.37 | L | 9 |

[a] H, histidine-citrate; L, lithium-borate; and M, morpholine-citrate.
[b] See Literature Cited section.

improve the intensity of the faintly staining enzymes. ALP and G6P produced dark, consistent bands that smeared and could not be interpreted; consequently, these assays were not used in the analysis.

Genetic interpretation of the detected isozymes was based on results from similar studies (4,10,14,17,21) and on the understanding that *P. harknessii* spores are dikaryotic (13). Migration distance and band morphology were used to characterize zones of activity as representative of individual gene loci and bands within each zone as allozymes (i.e., allelic differences in isozyme mobility). Confirmation of inheritance could not be made, however, because it is not yet possible to make control crosses among individual rust isolates.

Isozymes from seven of the examined enzymes were monomorphic in *P. harknessii*. These were: DIA-1, DIA-2, MDH, MNR, MPI, 6PG, PGM-1, and PGI (Fig. 2). Of these, DIA displayed two zones of activity that were widely separated from each other and never varied with respect to each other. These two zones were interpreted as two independent loci. PGM also exhibited two separate zones of activity; however, only PGM-1 was monomorphic. These zones were also interpreted as two independent loci. Multiple bands occurred within a single zone of activity for MPI. The relative mobility of these bands remained constant with respect to each other, and the intensity was equal among the three bands. Several alternative interpretations exist for the expression of MPI in *P. harknessii*. This pattern could be interpreted as: three independent monomorphic loci; two monomorphic loci with an interlocus heterodimeric band; single monomorphic locus with multiple bands from a single allele (i.e., post translational modification); a single monomorphic locus with secondary staining from an alternate enzyme; or nonvariant, heterozygous expression of a dimeric enzyme. Typically, a heterozygous dimeric enzyme would display as 1:2:1 ratio in band intensity. The equality of MPI bands and the nonvariant condition diminishes the likelihood of the fifth alternative. The similarity in band morphology reduces the likelihood that the three MPI bands were caused by activity from alternate enzymes. The designation of MPI expression as one, two, or three loci is not possible unless variability is detected for this enzyme within *P. harknessii*. Accordingly, MPI was interpreted as a single homozygous locus. Single bands within a single zone of activity, that is, single monomorphic loci, were displayed by MDH, MNR, 6PG, and PGI.

An isozyme was considered polymorphic when the most frequent allele was present less than 95% of the time. Of the examined enzymes there were five polymorphic isozymes in *P. harknessii*. ACP displayed two allozymes within a single zone of activity (Fig. 3). The most frequent allozyme occurred 91% of the time. CAT displayed three negative image allozymes within a single zone of activity. The most frequent expression occurred in 58% of the spore samples. GOT displayed three allozymes with three bands each within a single zone of activity. Within a single zone of activity, the three bands were of equal size and intensity, and were therefore considered as a single allozyme phenotype. Similar patterns for GOT have been reported for other species (16). The most frequent expression occurred in 57% of the spore samples. EST and PGM each displayed three allozymes within single zones of activity. The most frequent allozyme occurred 73% of the time in EST and 89% of the time in PGM-2. Based on a composite of the polymorphic isozymes, there were 25 distinct isozyme biotypes among the 166 samples. The most frequent distribution (ACP = 1/1, CAT = 1/1, EST = 1/1, GOT = 1/1, and PGM-2 = 1/1) occurred in 39% of the samples; the next most frequent (ACP = 1/1, CAT = 2/2, EST = 2/2, GOT = 2/2, and PGM-2 = 1/1) occurred in 6% of the samples. Conversely, 44% of all biotypes were represented by single isolates.

Enzyme phenotypes of *C. q. banksianae* were generally similar to those of *P. harknessii* (Figs. 4 and 5). The monomorphic isozymes DIA-1, DIA-2, MDH, MNR, MPI, 6PG, PGM-1, and PGI were identical to *P. harknessii*. In contrast, ACP and GOT were monomorphic in the sampled *C. q. banksianae* spores. This may be attributed to sampling error because fewer galls were examined in *C. q. banksianae*. The polymorphic isozymes, CAT, EST, and PGM-2, had allozyme variants similar to the polymorphic isozymes in *P. harknessii*. However, isozyme banding patterns typical of heterozygous systems were found in PGM-2 and

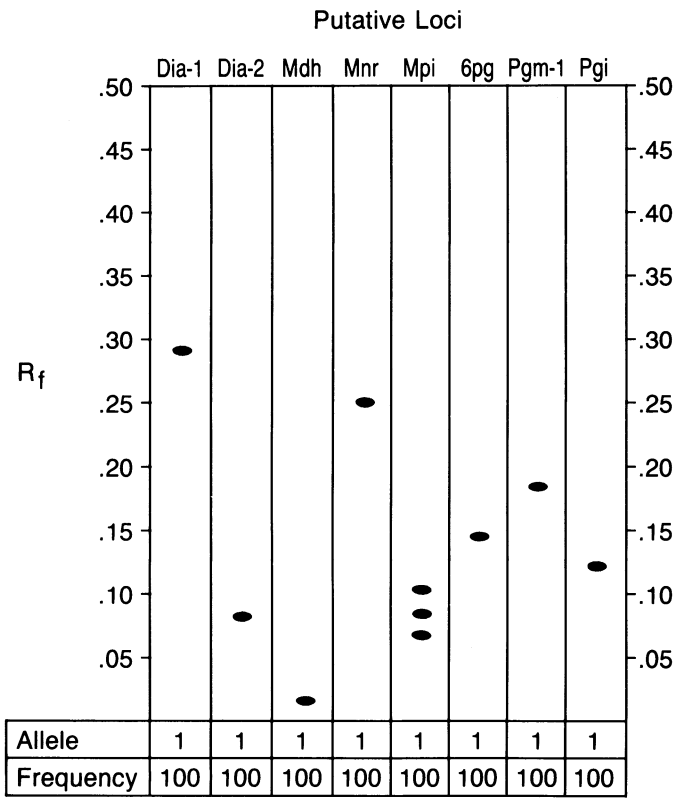


**Fig. 2.** Allele designations and relative mobilities of monomorphic isozymes of *Peridermium harknessii*.

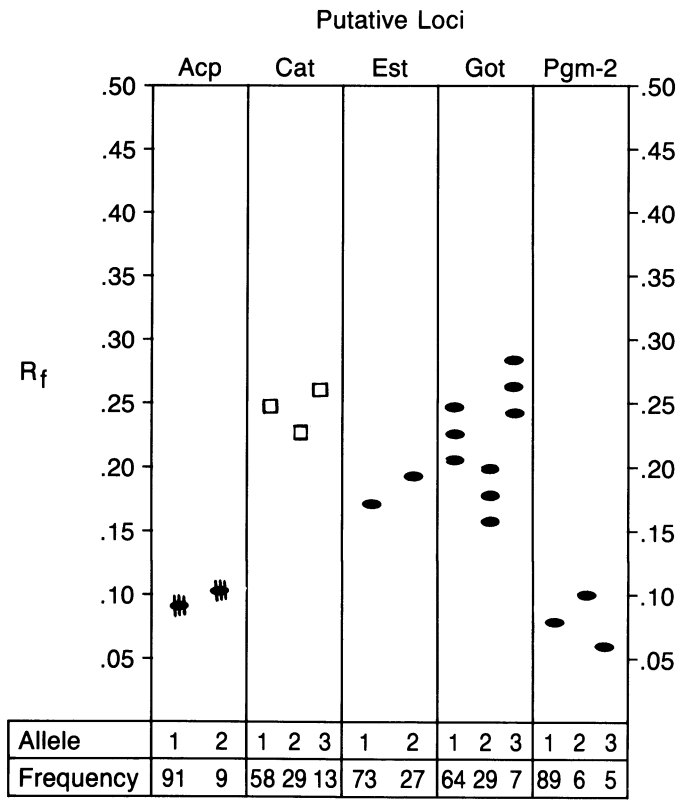


**Fig. 3.** Allele designations and relative mobilities of polymorphic isozymes of *Peridermium harknessii*.

EST. Note that Figure 4 depicts alternate allozymes and that heterozygous samples expressed a combination of two alternate alleles (e.g., PGM-2 = 1/2). Allozyme frequencies were significantly different between the two species for two of the three

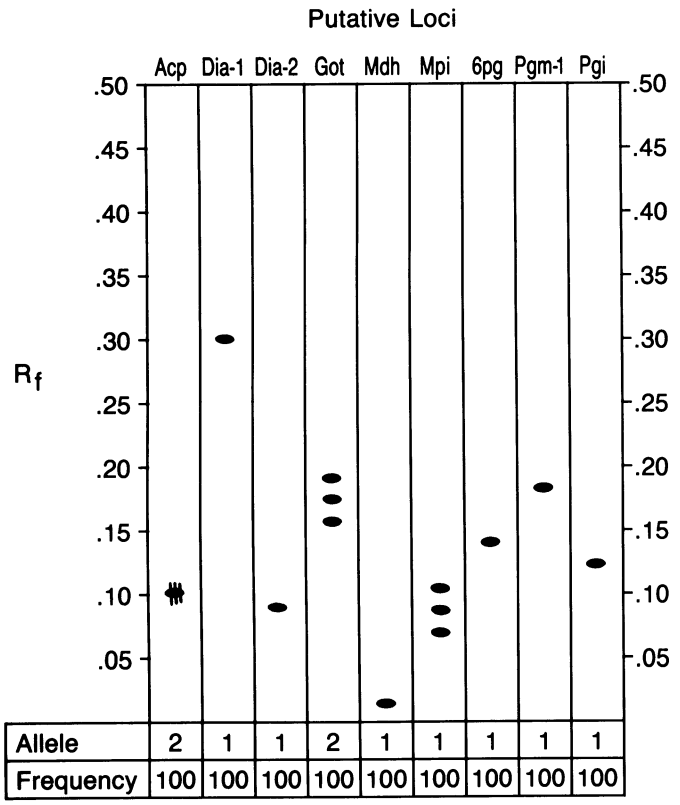


**Fig. 4.** Allele designations and relative mobilities of monomorphic isozymes of *Cronartium quercuum* f. sp. *banksianae*.

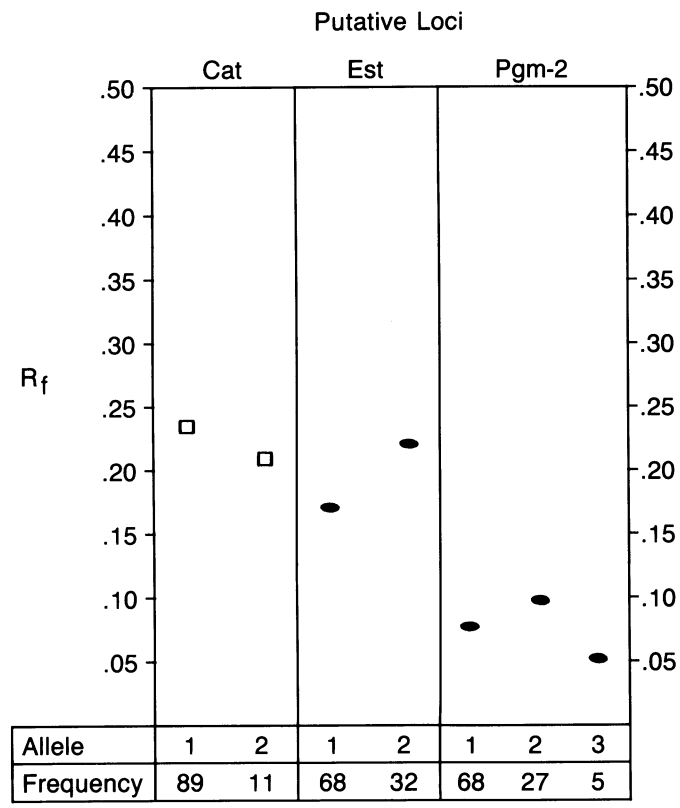


**Fig. 5.** Allele designations and relative mobilities of polymorphic isozymes of *Cronartium quercuum* f. sp. *banksianae*.

polymorphic isozymes (contingency Chi-square test, $X^2_{CAT}$ = 22.67**, $X^2_{EST}$ = 0.65, and $X^2_{PGM-2}$ = 12.96**), reflecting interspecific differences.

Based on a composite of the polymorphic loci, there were 11 distinct biotypes among the 31 samples of *C. q. banksianae*. The most frequent distribution (ACP = 2/2, CAT = 1/1, EST = 1/1, GOT = 2/2, and PGM-2 = 1/2) occurred in 26% of the samples. The next most frequent biotype (ACP = 2/2, CAT = 1/1, EST = 1/1, GOT = 2/2, and PGM-2 = 2/2) occurred in 16% of the samples. Conversely, 45% of all biotypes were represented by single isolates. The 11 *Cronartium* biotypes were not found among the 25 Peridermium biotypes.

Relationships between isozyme variability and pathogen virulence or host preference have been reported in other fungi (2,6,7,15). Furthermore, descriptions of the magnitude of genetic variation in the pathogen and inferences on mating systems and nuclear state have been gleaned from isozyme data (5,12,15,18). The electrophoretic technique described above appears to provide sufficient resolution to separate *P. harknessii* from *C. q. banksianae*. Spore samples from individual gall collections of *P. harknessii* appear to be homozygous, possibly homokaryotic. That is, if aeciospores of *P. harknessii* are dikaryotic, as reported by Epstein and Buurlage (13), and only single bands are detected for each polymorphic locus, then the nuclei of *P. harknessii* must be homozygous. The heterozygous banding patterns displayed in *C. q. banksianae* affirms the interpretation of the homozygous nature of *P. harknessii*.

These results indicate that starch gel electrophoresis can be used to examine variability in populations of *P. harknessii* and *C. q. banksianae*. The sampled population of *P. harknessii* appears to be heterogeneous and homozygous, and *C. q. banksianae* appears to be heterogeneous and heterozygous.

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