# Analysis of Allozymes of Three Distinct Variants of *Verticicladiella wageneri* Isolated from Conifers in Western North America

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## ABSTRACT

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Isolates representing the three variants of *Verticicladiella wageneri* from hard pine, Douglas-fir, and pinyons were subjected to isozyme analysis by starch gel electrophoresis of mycelial extracts. Nine allozymes distributed among 10 putative loci produced scoreable banding patterns. From the resultant genotypic information, allele frequencies and genetic distance measures were calculated. Seven of the 10 loci were monomorphic across all variants, indicating a degree of genetic uniformity in the fungus. The magnitude of the differences between the pinyon variant and the others is reflected in apparent fixation for alternate alleles between these variants in the isocitrate dehydrogenase locus and between the pinyon and the hard pine variant for the glutamate-oxaloacetate transaminase locus. The genetic divergence of the pinyon isolates indicated by the isozyme data correspond to geographic and ecological isolation of the host. The designation of a third variety of *V. wageneri* delimiting the Douglas-fir variant is suggested.

*Additional key words*: *Ceratocystis wageneri*, electrophoresis, isozymes, population genetics, root disease fungi.

Black-stain root disease, caused by *Verticicladiella wageneri* Kendr., is a serious problem in Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), hard pines (*Pinus ponderosa* Laws., *P. contorta* Dougl., and *P. jeffreyi* Grev. & Balf.), and pinyons (*P. edulis* Engelm. and *P. monophylla* Torr. et Frem.) in the western United States. Isolates from these hosts can be easily distinguished in culture by their characteristic early pigmentation, amount of sporulation, and colony margin characters (8,10). Host specialization experiments with isolates from these three host groups, primarily with greenhouse seedlings, have confirmed these distinctions (9); however, microscopic studies have failed to yield sufficient morphological differences to separate Douglas-fir isolates from pinyon isolates (10). Thus, those authors (10) named only two varieties, var. *ponderosa* for isolates from hard pines and var. *wageneri* for Douglas-fir and pinyon isolates. Further information clarifying the relationships among the three variants of this pathogen is therefore desirable.

Isozyme techniques have only recently been used to study population genetics of plant-pathogenic fungi (3). Bonde et al (2) applied isozyme analysis to distinguish species of *Peronosclerospora* and to provide information on their phylogenetic relationships. Old et al (15) used this approach to study genetic variability in isolates of *Phytophthora cinnamomi* Rands from Australia and Papua New Guinea. They showed that isozyme markers could be of value as a diagnostic tool to distinguish among *Phytophthora* isolates and to study evolutionary relationships among species of the genus. Other studies of isozymes in plant-pathogenic fungi have provided the necessary genetic information to separate formae speciales in wheat and barley yellow rusts (14).

There is currently no information available regarding use of isozyme analyses in the study of the population structure of important forest tree pathogens such as *V. wageneri*. Such information can provide insights into taxonomic and pathological relationships of this pathogen and contribute to the resolution of the taxonomic hiatus among the three variants of this fungus. The purpose of this study, therefore, is to use isozyme analysis to provide preliminary information on the nature of genetic variability in *V. wageneri* and to determine genetic relationships among the three variants.

## MATERIALS AND METHODS

Twenty-six isolates of *V. wageneri* (10 from hard pines, 10 from Douglas-fir, and 6 from pinyons) representing the three variants defined in an earlier study by Harrington and Cobb (10) were obtained. Details on their host and geographic origin are given in Table 1. Isolates were hyphal-tipped onto PDA plates and

incubated at 18 C for 2 wk. Agar plugs from the actively growing margins of each isolate were taken with a sterile 4-mm cork borer and transferred to 100 ml of sterile potato-dextrose broth (Difco) prepared in 250-ml Erlenmeyer flasks. All inoculated flasks were incubated at 18 C for 2 wk and gently swirled for a few seconds every 3–4 days. Preliminary studies indicated that incubating cultures for 2 wk yielded optimal staining intensity for all enzyme systems. Staining intensity decreased markedly after 3 wk of incubation.

After incubation, mycelial mats from each isolate were harvested in a Buchner funnel by vacuum filtration over Whatman No. 1 filter paper disks. The mats were gently rinsed with 30–50 ml of distilled water and allowed to drain over gentle vacuum until excess water was drawn off. Portions of mycelium from each isolate were placed in small 0.3-ml wells drilled into a Lucite block (6). About one-third of the volume of each well was filled with mycelium (16). Blocks containing the mycelium were then tightly wrapped with plastic film, placed in a freezer, and stored at −60 C for 2 or 3 days before extraction and electrophoresis.

Starch gel slabs were prepared before mycelial extraction according to the method of Conkle et al (6). The four gel buffer systems used in all electrophoresis runs were Tris-citrate (pH 8.8 and pH 8.3) and morpholine-citrate (pH 6.1 and pH 8.1) (Table 2). Enzyme extraction was carried out by adding about 0.1 ml of chilled 0.2 M phosphate buffer, pH 7.5, amended with 40 mg of bovine serum albumin and one drop of mercaptoethanol per 100 ml of buffer to each well. The buffered mycelium in each well was crushed with a 3-mm-diameter glass rod for about 20 sec. The blocks were kept chilled on a cold pack during extraction. Filter paper wicks made from Whatman chromatography paper cut to 2 × 10 mm were placed in each well to absorb the extract. Wicks were gently blotted with absorbent tissue, then transferred to their appropriate tracks on the horizontal starch gel slabs. Electrophoresis was performed in refrigerated compartments with cold water bags on top of the gels to prevent overheating. Specific operational parameters given by Conkle et al (6) were followed with respect to length of electrophoresis time, electrical current applied, and staining procedures for various enzyme systems. For one enzyme system, uridine diphosphoglucose pyrophosphorylase (UGPP), the staining procedure of Harris and Hopkinson (12), was used.

Because the fungus does not form the perfect state in pure culture, the genetic basis of the isozyme bands, or genotype, cannot be unequivocally determined. Therefore, we elected to take a conservative approach to the interpretation of observed isozyme phenotypes by assuming mobility differences, or electromorphs, are allelic variants within a given isozyme locus. Subsequent references to alleles or allele frequencies are used in this sense.

Presumed genotypes were recorded by assigning an integer value starting with 1 for the most common allele, 2 for the next most common, etc., of a putative locus coding for an enzyme system. In a case where two loci appeared to be coding for a given enzyme, the locus with the most anodal mobility was given the number 1 after the name of the enzyme system, and the next most mobile was given the number 2. The enzyme systems analyzed and the gel buffers used (6) are given in Table 2. Maps representing all positions of the isozyme bands relative to the origin and buffer front were prepared to permit comparisons between runs on different gels. Two separate electrophoresis runs were performed for all isolates.

Allele frequencies were determined and cluster analysis was performed using the unweighted pair group method (18) based on Cavalli-Sforza and Edwards's (5) chord distance.

## RESULTS

Nine enzyme systems representing 10 putative loci were found to have readily interpretable banding patterns (Table 2). Allele frequencies for each variable locus and fungal variant are presented in Table 3. One isolate from a Douglas-fir variant did not produce a band for the GOT locus; therefore, analyses were based on nine isolates for that variant locus.

A diagrammatic representation of the relative mobilities of the variable allozymes is given in Figure 1. The IDH locus was interpreted as having two alleles, each having three bands. Staining intensity was equal for all bands in this locus, and no band had the same mobility between each allele. Although IDH reportedly has a dimeric subunit structure (12) and the triple band is reminiscent of the banding pattern expected with a heterozygous, dimeric allozyme, the haplontic life cycle of this fungus and other Ascomycetes precludes this interpretation.

The G6PD locus also had three bands. The two bands having the greatest anodal mobility were invariant for all isolates and were assumed to be products of one locus, although it is possible that two loci are involved. The variable band types were interpreted as alleles of the G6PD2 locus because they had mobilities independent of G6PD1. Multiple banding patterns occurring in these systems can also result from enzymes having cofactor-bound

TABLE 1. Hosts and geographic origins of *Verticicladiella wageneri* isolates used in isozyme study

| Variant and isolate no. | Host or origin | Location |
|---|---|---|
| Hard pine | | |
| BCL-1 | *Pinus contorta* | Monashee, BC |
| CAJ-3 | *P. jeffreyi* | Blacks Mtn., CA |
| CAP-1 | *P. ponderosa* | Gaddis Creek, CA |
| CAP-19 | *P. ponderosa* | McCloud Flat, CA |
| CAP-36 | *P. ponderosa* | Sugar Pine Creek, CA |
| CAP-H | *P. ponderosa* | Butcher Corral, CA |
| IDP-1 | *P. ponderosa* | Boville, ID |
| MOW-1 | *P. monticola* | Yaak River, MT |
| ORH-1 | *Tsuga heterophylla* | Mt. Hood, OR |
| ORL-1 | *P. contorta* | Sisters, OR |
| Douglas-fir | | |
| BCD-1 | *Pseudotsuga menziesii* | Cicero, BC |
| BCH-1 | *T. heterophylla* | Tugwell, BC |
| CAD-1 | *P. menziesii* | Rock Creek, CA |
| CAD-7 | *P. menziesii* | Sugar Pine Creek, CA |
| CAD-18 | *P. menziesii* | Union Valley, CA |
| CAD-21 | *P. menziesii* | Trinidad, CA |
| CAD-32 | *P. menziesii* | Greagle, CA |
| MOD-1 | *P. menziesii* | Flathead Lake, MT |
| NMD-1 | *P. menziesii* | Mescalero, NM |
| ORD-5 | *P. menziesii* | Burnt Mtn., OR |
| Pinyon | | |
| CAS-1 | *Pinus monophylla* | Onyx Summit, CA |
| CAS-3 | *P. monophylla* | Big Bear Lake, CA |
| CAS-4 | *P. monophylla* | Kennedy Meadows, CA |
| CAS-5 | *P. monophylla* | Kennedy Meadows, CA |
| COE-1 | *P. edulis* | Mesa Verde, CO |
| NME-1 | *P. edulis* | Jicarilla, NM |

TABLE 2. Enzyme systems from *Verticicladiella wageneri* variants and gel buffer systems yielding readable bands after starch gel electrophoresis

| Enzyme system | EC number | Abbreviation | Gel buffer system[y] |
|---|---|---|---|
| Menadione reductase | 1.6.99.2 | MNR | A |
| Leucine aminopeptidase | 3.4.11.1 | LAP | D |
| Peptidase | 3.4.13.1 | PEP | A |
| Glutamate dehydrogenase | 1.4.1.2 | GDH | B |
| Glutamate oxaloacetate transaminase | 2.6.1.1 | GOT | B |
| Glucose 6-phosphate dehydrogenase | 1.1.1.49 | G6PD | B |
| Uridine diphosphoglucose pyrophosphorylase | 2.7.7.9 | UGPP | B[z] |
| Isocitrate dehydrogenase | 1.1.1.42 | IDH | D |
| Malic dehydrogenase | 1.1.1.37 | MDH | E |

[y] After Conkle et al (6). The four buffer systems used were Tris-citrate (pH 8.8 and 8.3) and morpholine citrate (pH 8.1 and pH 6.1), abbreviated A, B, D, and E, respectively.

[z] After Harris and Hopkinson (12).

and unbound forms present, from posttranslational modification of the enzymes or from other nongenetic factors.

Nevertheless, a low degree of polymorphism is evident for these allozymes because GOT, G6PD2, and IDH were the only variable loci. The mean number of alleles per locus and standard errors in parentheses for the hard pine, Douglas-fir, and pinyon pine variants are 1.1 (0.1), 1.2 (0.2), and 1.0 (0.0), respectively.

The hard pine and the pinyon isolates are fixed for alternate alleles in GOT and IDH loci, whereas the Douglas-fir isolates show some degree of polymorphism in the GOT and G6PD2 loci. In general, the hard pine and Douglas-fir isolates have more alleles in common among the three variable loci than do the pinyon isolates.

The genetic distance coefficients (5) among all comparisons of the three variants of *V. wageneri* are given in Table 4. The values range from 0, when all alleles in the population are shared, to 1, when the populations have no alleles in common. The pinyon isolates had the greatest distance value relative to both the hard pine and Douglas-fir isolates.

## DISCUSSION

The variants of *V. wageneri* were monomorphic for seven of 10 loci examined. This low amount of variation is in contrast to the high degree of allozyme polymorphism reported for *Fomes annosus* isolates from both pine and true fir (*Abies*) hosts (17). The enzymes used in the present study are those having resolvable bands, and they are assumed to be representative of the genome with respect to allelic variation (1). Not all allelic variability is detected by electrophoresis, and the amount of variability and genetic differentiation between groups may be underestimated (1,13).

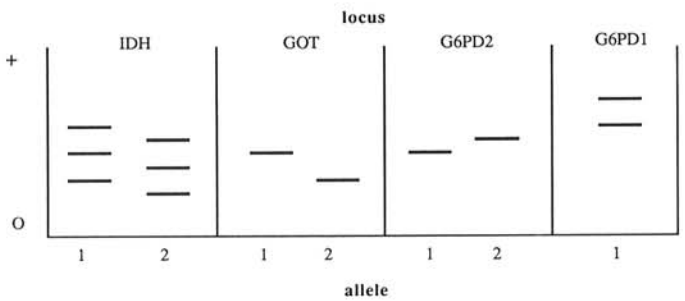


**Fig. 1.** Diagram showing the banding patterns of the variable isozyme loci IDH, GOT, and G6PD occurring in *Verticicladiella wageneri*. G6PD1 is shown to illustrate band mobility relative to G6PD2. Direction of migration is toward anode (+) from origin (0) for all bands.

TABLE 3. Allele frequencies of variable loci among three variants of *Verticicladiella wageneri*

| Locus (allele) | | Allele frequency of variant: Hard pine (10 isolates) | Douglas-fir (10 isolates) | Pinyon (six isolates) |
|---|---|---|---|---|
| GOT | (1) | 1.00 (0.67–1.00)[a] | 0.78 (0.42–0.96) | 0.00 (0.00–0.47) |
| | (2) | 0.00 (0.00–0.30) | 0.22 (0.03–0.57) | 1.00 (0.50–1.00) |
| G6PD2 | (1) | 0.40 (0.13–0.74) | 0.90 (0.56–1.00) | 1.00 (0.50–1.00) |
| | (2) | 0.60 (0.27–0.87) | 0.10 (0.02–0.43) | 0.00 (0.00–0.47) |
| IDH | (1) | 1.00 (0.67–1.00) | 1.00 (0.67–1.00) | 0.00 (0.00–0.47) |
| | (2) | 0.00 (0.00–0.30) | 0.00 (0.00–0.30) | 1.00 (0.50–1.00) |

[a] Numbers in parentheses are 95% confidence limits.

TABLE 4. Chord distance coefficients[a] based on allele frequencies between all pairwise comparisons of three variants of *Verticicladiella wageneri*

| Morphological variants compared | Distance coefficient |
|---|---|
| Hard pine × Douglas-fir | 0.149 |
| Hard pine × pinyon | 0.438 |
| Douglas-fir × pinyon | 0.358 |

[a] From Cavalli-Sforza and Edwards (5).

The apparent genetic uniformity as reflected by a low level of allozyme polymorphism may be a general characteristic of this fungus as well as other highly specialized biotrophic fungi (14). Unfortunately, there are no comparative studies of genetic variation in fungi having biotrophic, necrotrophic, or saprotrophic nutrition. Environmental heterogeneity may be important in maintaining allozyme polymorphism in a saprophytic fungus like *Neurospora intermedia* (19), whereas *V. wageneri* would be somewhat shielded from environmental variations as a consequence of its etiology. One insect vector of *V. wageneri* in pine, *Hylastes macer*, is reportedly specific to *Pinus* spp. (7) but has not been reported on the pinyons. *H. nigrinus* has been shown to vector *V. wageneri* in thinned stands of Douglas-fir in California (11,21), but its specificity to Douglas-fir is uncertain (20,22).

Another factor that could contribute to the observed allozyme uniformity in *V. wageneri* is the apparent rarity of a sexual stage. Goheen and Cobb (7) described the teleomorph, *Ceratocystis wageneri*, in galleries of *H. macer* in infected roots of ponderosa pine. However, there has been no report of the perfect state in either Douglas-fir or pinyons and no other such report on hard pines. Based on isozyme analysis, asexually reproducing populations of *Puccinia graminis* f. sp. *tritici* were found to be genetically less diverse than sexually reproducing populations (4).

Allozyme differentiation exists among the three variants, particularly with regard to the pinyon isolates. The six isolates from pinyon were from New Mexico, Colorado, and California, and the lack of variability in all 10 loci in this group is evidence that one genotype may be predominant. The degree of differentiation observed in the pinyon variant of *V. wageneri* may be due to the geographic or ecological isolation of its host and possibly unknown host specific insect vectors. Such isolation has resulted in significant differences in allele frequencies between allozymes of the A1 mating types of *Phytophthora cinnamomi* from Australia and Papua New Guinea (15). Differences in allele frequencies of allozymes of *N. intermedia* were also found to increase with geographic separation (19).

The genetic distance between the pinyon isolates on one hand and the Douglas-fir and hard pine isolates on the other (Table 4) may represent a relatively sizable phylogenetic separation between these groups. This is also reflected in the distinct colony morphologies, differential sensitivity to temperatures above 25 C, and host specificity among the three variants (8,9). Ayala et al (1) describe population genetic studies of allozyme variation in *Drosophila* species over wide geographic areas where genetic distance values related to those reported in this study are of the magnitude differentiating subspecies or semispecies. Very little comparative population genetics information has been reported for plant-pathogenic fungi. Bonde et al (2), in their study of isozymes of 10 isolates representing three species of *Peronosclerospora*, were able to separate species of this fungus in their calculations of allele frequencies and coefficients of similarity. Their isozyme data also provided evidence that certain species may be misidentified and suggested taxonomic reevaluation of the genus. Newton et al (14) compared isozyme bands between *Puccinia striiformis* derived from wheat yellow rust and those of barley yellow rust. Although these rust strains are morphologically similar, isozyme and host specificity evidence warranted the recognition of these two rust groups as formae speciales.

Harrington and Cobb (10) recently separated the hard pine variants from the Douglas-fir and pinyon variants by creating two new varieties. One variety, var. *ponderosae*, is represented by the hard pine isolates and the other variety, var. *wageneri*, represents Douglas-fir and pinyon isolates. They cited the lack of a definitive microscopic character separating the latter variants, although acknowledging that combinations of characteristics such as sensitivity to temperatures above 25 C, width and color of colony margins, and amount of sporulation in culture suggest two distinct populations. Earlier experiments employing the isolates analyzed in our study have demonstrated host specificity between the

Douglas-fir and the hard pine variants (9). Field observations and more recent inoculation studies also suggest host specificity among the pinyon, hard pine, and Douglas-fir variants (F. W. Cobb, *unpublished*). In our study, the pinyon isolates differed in their isozymes to the extent that they appeared conspicuously apart from the other two variants, as reflected in their genetic distances (Table 4). There is evidence that genetic differentiation at the molecular level can take place in fungi before morphologically distinguishable characters appear (2,14–16). The isozyme data reported here and the pathological and morphological relationships defined in earlier studies suggest that designation of a third variety of *V. wageneri*, delimiting the Douglas-fir variant, should be considered.

## LITERATURE CITED


1. Ayala, F. J., Tracey, M. L., Hedgecock, D., and Richmond, R. C. 1974. Genetic differentiation during the speciation process in *Drosophila*. Evolution 28:576-592.
2. Bonde, M. R., Peterson, G. L., Dowler, W. M., and May, B. 1984. Isozyme analysis to differentiate species of *Peronosclerospora* causing downy mildews of maize. Phytopathology 74:1278-1283.
3. Burdon, J. J., and Marshall, D. R. 1983. The use of isozymes in plant disease research. In: Isozymes in Plant Genetics and Breeding. Part A. S. D. Tanksley and T. V. Orton, eds. Elsevier, Amsterdam.
4. Burdon, J. J., and Roelfs, A. P. 1985. The effect of sexual and asexual reproduction on the isozyme structure of populations of *Puccinia graminis*. Phytopathology 75:1068-1073.
5. Cavalli-Sforza, L. L., and Edwards, A. W. F. 1967. Phylogenetic analysis: Models and estimation procedures. Am. J. Human Genet. 19:233-257.
6. Conkle, M. T., Hodgskiss, P. D., Nunnally, L. B., and Hunter, S. C. 1982. Starch gel electrophoresis of conifer seeds: A laboratory manual. U.S. Dep. Agric. For. Serv. Gen. Tech. Rep. PSW-64. 18 pp.
7. Goheen, D. J., and Cobb, F. W. 1978. Occurrence of *Verticicladiella wagenerii* and its perfect state, *Ceratocystis wageneri* sp. nov. in insect galleries. Phytopathology 68:1192-1195.
8. Harrington, T. C., and Cobb, F. W. 1981. Infra-specific variants of *Verticicladiella wageneri*. (Abstr.) Phytopathology 71:879.
9. Harrington, T. C., and Cobb, F. W. 1984. Host specialization of three morphological variants of *Verticicladiella wageneri*. Phytopathology 74:286-290.
10. Harrington, T. C., and Cobb, F. W. 1986. Varieties of *Verticicladiella wageneri*. Mycologia 78(4):562-567.
11. Harrington, T. C., Cobb, F. W., and Lownsbery, J. W. 1985. Activity of *Hylastes nigrinus*, a vector of *Verticicladiella wageneri*, in thinned stands of Douglas-fir. Can. J. For. Res. 15:519-523.
12. Harris, H., and Hopkinson, O. A. 1976. A Handbook of Enzyme Electrophoresis in Human Genetics (with supplements). North Holland Publishing Company, Amsterdam.
13. Johnson, G. B. 1977. Assessing electrophoretic similarity: The problem of hidden heterogeneity. Annu. Rev. Ecol. Syst. 8:309-328.
14. Newton, A. C., Caten, C. E., and Johnson, R. 1985. Variation for isozymes and double-stranded RNA among isolates of *Puccinia striiformis* and two other cereal rusts. Plant Pathol. 34:235-247.
15. Old, K. M. Moran, G. F., and Bell, J. C. 1984. Isozyme variability among isolates of *Phytophthora cinnamomi* from Australia and Papua New Guinea. Can. J. Bot. 62:2016-2022.
16. Otrosina, W. J. 1985. Isozyme polymorphism in *Fomes annosus*. Pages 97-99 in: Proc. Annu. West. Int. For. Dis. Work Conf. 33rd. Olympia, WA.
17. Otrosina, W. J. 1986. Allozyme relationships between isolates of *Fomes annosus* from pine and true fir in California. (Abstr.) Phytopathology 76:1112.
18. Sneath, P. H. A., and Sokal, R. R. 1973. Numerical Taxonomy. W. H. Freeman & Company, San Francisco. 574 pp.
19. Speith, P. T. 1975. Population genetics of allozyme variation in *Neurospora intermedia*. Genetics 80:785-805.
20. Witcosky, J. J., and Hansen, E. M. 1985. Root-colonizing insects associated with Douglas-fir in various stages of decline due to black-stain root disease. Phytopathology 75:399-402.
21. Witcosky, J. J., Schowalter, T. D., and Hansen, E. M. 1986. The influence of time of precommercial thinning on the colonization of Douglas-fir by three species of root-colonizing insects. Can. J. For. Res. 16:745-749.
22. Wood, F. L. 1982. The Bark and Ambrosia Beetles of North and Central America (Coleoptera: Scolytidae). A Taxonomic Monograph. Great Basin Naturalist Memoirs No. 6. Brigham Young University Press. 1,359 pp.