

## **Chapter 9**

# **Isozymes: Methods and Applications\***

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### I. INTRODUCTION

Isozyme analysis is a powerful biochemical technique with numerous applications in plant pathology. It has long been used by geneticists to study the population genetics of fish, mammals, insects, nematodes, and higher plants. Mycologists and plant pathologists more recently adopted the procedure, and it is now being used routinely to settle taxonomic disputes, identify “unknown” cultures, “fingerprint” patentable fungal lines and plant cultivars, analyze genetic variability, trace pathogen spread, follow the segregation of genetic loci, and determine ploidy levels of fungi and other plant pathogens. These topics have been recently reviewed.<sup>1,2</sup> The large number of publications in this field each year indicates the widespread interest in isozyme analysis.

In this paper, we discuss some major applications of isozyme analysis in basic and applied plant pathology. The technique is particularly useful with fungi; the greatest advances have been mostly with fungal pathogens. Isozyme banding patterns obtained from fungi are usually relatively uncomplicated and easy to interpret. Isozyme analysis can be readily performed in most laboratories with relatively little expense. With the development of computer programs that enable large numbers of comparisons at the gene level, much information can be obtained about the population genetics and life cycle of the organism. Isozyme analysis has proven particularly useful in situations where it is necessary to differentiate among two or more morphologically similar fungi. These and other uses for isozyme analysis will be discussed, along with advantages and disadvantages as compared to alternative techniques.

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## II. PRINCIPLES

Isozymes are defined as multiple molecular forms of a single enzyme. These forms usually have similar, if not identical, enzymatic properties, but slightly different amino acid compositions due to differences in the nucleotide sequence of the DNA that codes for the protein. Often the only difference among isozymes is the substitution of one to several amino acids.

Only those isozymes that have large variations in size or shape or that differ in net charge can be separated by electrophoresis. Differences in net charge can occur when a basic amino acid, such as lysine, is substituted for an acidic amino acid, such as aspartic acid. Only 28.7% of all amino acid substitutions will change the net charge of a protein.<sup>3</sup> Some amino acid substitutions that do not involve charge differences can also affect the electrophoretic mobility of a protein, presumably by altering the tertiary structure of the enzyme.<sup>4</sup> Thus, about one third of all single amino acid substitutions will be electrophoretically detectable, and several simultaneous substitutions can cancel out the effect. Isozyme analysis therefore provides a very conservative estimate of the extent of genetic variability within a population.<sup>3</sup>

Detectable isozymes can arise from three different genetic and biochemical conditions: (1) multiple alleles at a single locus, (2) single or multiple alleles at multiple loci, and (3) secondary isozymes, usually arising from post-translational processing.

### A. MULTIPLE ALLELES AT A SINGLE LOCUS

In a fungal population, any given genetic locus can be monomorphic (i.e., expresses a single allele in 99% or more of the population) or polymorphic (i.e., expresses more than one allele in 99% of the population). When a genetic locus is polymorphic, the isozymes formed by the expression of the different alleles are termed "allozymes". Each allele codes for a structurally distinct version of a particular polypeptide chain. The primary structure of an allozyme therefore depends on the number of alleles present and their nucleotide sequences. The number of alleles in any organism varies with its nuclear condition (monokaryotic, dikaryotic), ploidy number (haploid, diploid, polyploid), and genetic makeup (homozygous, heterozygous). The allozymes of individuals that are haploid or homozygous produce simple electrophoretic banding patterns due to the expression of a single allele. Allozymes of organisms that are diploid or dikaryotic and heterozygous produce more complex banding patterns due to the expression of two separate alleles.

Enzymes can consist of one or more polypeptide chains. Monomeric enzymes consist of a single polypeptide chain; multimeric (or oligomeric) enzymes are comprised of two or more polypeptide chains. Most multimeric enzymes are either dimeric (two chains) or tetrameric (four chains). The electrophoretic banding pattern obtained for monomeric enzymes is usually simple and easy to interpret even if the organism is heterozygous (Figure 1). Each allele is expressed as a single polypeptide band. The heterozygous condition appears as a mixture of isozymes produced by the two corresponding homozygotes.

More complicated patterns are formed in heterozygotes when the enzymes are multimeric due to the formation of intermediate, "heteromeric" (or hybrid) bands (Figure 1). These are in addition to the two "homomeric" forms associated with each homozygote. For example, if allele A codes for polypeptide a, and allele A' codes for polypeptide a', the following allozymes will be formed for a tetrameric enzyme: aaaa (homomeric), a'a'a'a' (homomeric), aaaa' (heteromeric), aaa'a' (heteromeric), and aa'a' a' (heteromeric). Other examples of heteromeric band formation for a single locus are detailed in Figures 1 and 2.

The frequency of occurrence of the forms of multimeric enzymes, assuming completely random combination of the polypeptide chains, should follow Mendelian ratios. The ratio of all possible isozymes formed in heterozygotes (assuming two alleles in the population) is 1:1 for monomers, 1:2:1 for dimers, 1:3:3:1 for trimers, and 1:4:6:4:1 for tetramers (heteromeric bands are boldfaced) (Figure 1). The relative quantities of the different isomers can often be recognized by differences in staining intensity; the heteromeric bands, which have a higher probability of being formed and are therefore present in larger quantities, should stain darker or more intensely. In some instances, a particular polypeptide will not contribute equally to the activity of the enzyme due to a slower rate of synthesis, low stability, or a tendency to break down before it can be assembled into the final enzyme. Certain polypeptide chains may reduce the activity of an enzyme by decreasing its stability or by reducing its catalytic ability. In such cases, the enzymes would not be detected in the expected ratios of staining intensity on the gel.

Extremely complex banding patterns may be obtained if three or more alleles are present in a population (Figure 2). Numerous heteromeric bands may be resolved. In most cases, the interpretation of such complex banding patterns should be confirmed by comparison to crosses of known genotypes: otherwise, readers and editors will be skeptical that the data were interpreted properly. The polypeptide composition of many enzymes is often conserved among organisms.<sup>6,7</sup> The genetic interpretation of banding patterns is much simpler when the polymeric composition of the enzyme is known.

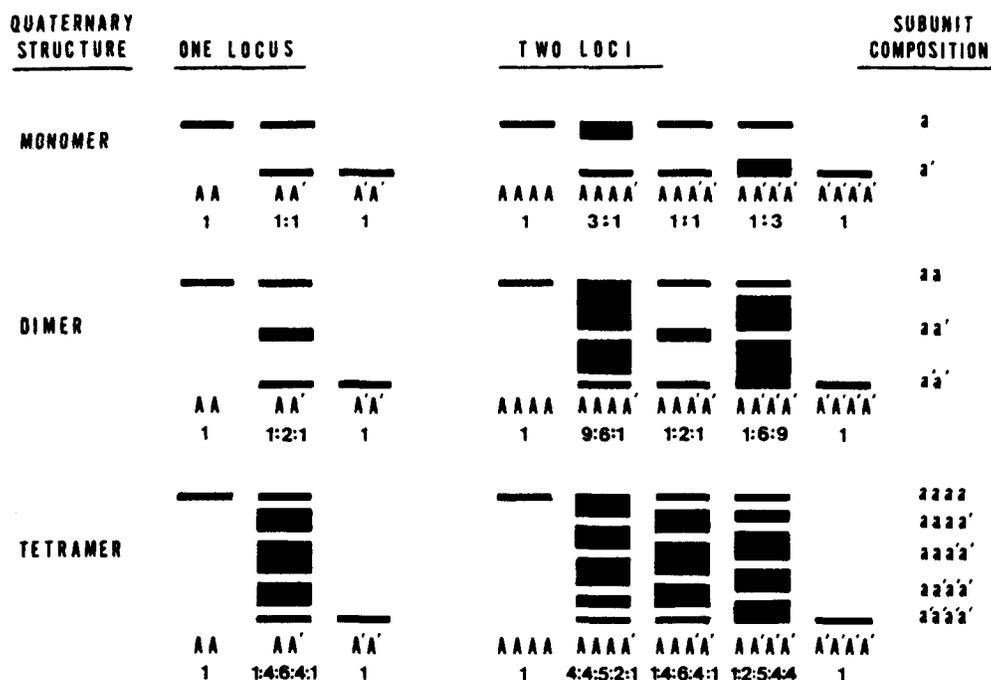


Figure 1 Predicted banding patterns for one locus with two alleles (A and A') and two segregating loci that share the same alleles for monomeric, dimeric, and tetrameric enzymes of a diploid or dikaryotic organism. Genotypes (in capital letters) are listed below each banding pattern. Subunit composition of each protein band is shown on right; lowercase letters represent subunit designations. The expected ratios of banding intensity for each phenotype is presented beneath the genotype. (Adapted from May, Reference 5.)

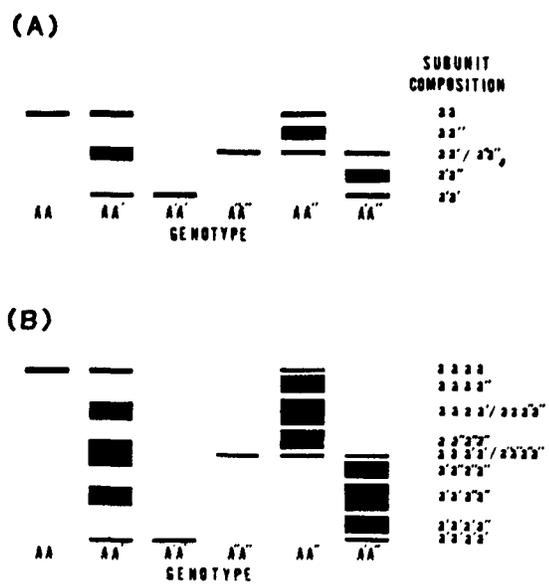


Figure 2 Predicted banding patterns of dimeric (A) and tetrameric (B) enzymes coded by a single locus and three electrophoretically distinct alleles (A, A', and A'') in a diploid or dikaryotic organism. Abbreviations as in Figure 1. (Adapted from May, Reference 5.)



Proteins may aggregate in a sample, especially if the pH or the ionic strength of the buffer is incorrect. Protein aggregation will result in poor resolution and uninterpretable banding patterns. Several different buffers should be tried during a preliminary “screening” run to determine which buffers prevent aggregation and provide the best resolution for a particular enzyme.

### III. METHODOLOGY

#### A. SAMPLE SELECTION AND PREPARATION

Sample selection and preparation are the most critical steps in isozyme analysis. The quality of genetic information obtained from an experiment is only as good as the sampling of isolates from which the data are derived. The number of isolates and their geographic and host range will all affect data interpretation. Care in sample preparation is also essential for a successful study. Poor resolution, faint staining or absence of bands, and irregular banding patterns can be caused by the incorrect choice of sample buffer or improper extraction techniques. Sample preparation has been thoroughly discussed elsewhere.<sup>1,2</sup>

The specific activity stains used in isozyme analysis detect only active enzymes; denaturation must be prevented during and after sample preparation. Samples should be kept cold (below 4°C) during preparation and storage. They can often be frozen at -80°C for up to 1 year, but this can vary for different organisms. Repeated freezing and thawing will result in denaturation. Enzymes may aggregate or precipitate if they are in concentrations that are too high or low or if the sample buffer is of the wrong pH or ionic strength. A commonly used sample buffer is Tris-HCl (0.01 to 0.1 M pH 6.8 to 7.5). Chelating agents, protease inhibitors, and enzyme stabilizers, such as EDTA, polyvinylpyrrolidone, PMSF, and dithiothreitol, can be added to the sample buffer to increase resolution. *Note: many of these reagents are extremely toxic. Use proper safety precautions to avoid contact with these compounds.* 2-Mercaptoethanol (20 µl/100 ml sample buffer) and bovine serum albumin (4 mg/ml) can also improve resolution by reducing the effects of resins, phenolics, and free fatty acids. Such contaminants are usually more of a problem when plant tissue is being extracted, although some fungal pigments fall into this category. The addition of small quantities of substrate (20 mg/100 ml sample buffer) may also help to stabilize some enzymes. Several substrates can be incorporated into a single sample buffer as long as they do not interact with each other. Alternatively, substrates can be added to the gel or electrode buffer, although this is generally not as effective.<sup>9</sup>

#### B. ELECTROPHORETIC TECHNIQUES

Different electrophoretic techniques can be used to separate isozymes, including starch gel electrophoresis, polyacrylamide gel electrophoresis (PAGE), isoelectric focusing, and two-dimensional electrophoresis. Advantages, disadvantages, and protocol references for different electrophoretic procedures have been summarized.<sup>2</sup> Traditionally, isozyme analysis was performed with starch or PAGE, but isoelectric focusing is now being used more commonly. Isoelectric focusing and two-dimensional electrophoresis resolve larger numbers of isozymes than do the other techniques, but the electrophoretic banding patterns may not lend themselves to a genetic interpretation due to their complexity.

The identification and visualization of individual enzymes using specific activity stains requires the presence of active, nondenatured enzymes. Dissociating procedures, such as SDS-PAGE (polyacrylamide gel electrophoresis in which the proteins are denatured with the detergent sodium dodecyl sulfate), cannot be used for isozyme analysis.

#### C. STAINING

Isozymes are visualized on the electrophoretic gel by reaction with specific activity stains. Detection of specific enzymes is possible because the appropriate substrates and cofactors required for activity are provided in the staining solution. The enzymatic reaction forms a colored product, either through direct activity with a dye or by involving other enzymes in a series of reactions with the generation of a colored product as a final result. For some enzymes, such as superoxide dismutase, isozymes are seen as white bands on a dark background. Fluorescent products can be detected with ultraviolet light. Conversely, non fluorescent products can be visualized as “negatively stained” by reacting the starch with a fluorescent compound. The biochemistry of the different staining reactions has been discussed,<sup>6,10</sup> and stain “recipes” for many different enzymes have been described.<sup>6,9,11-15</sup>

The selection of enzymes to study is an important part of isozyme analysis and can have a dramatic impact on the results and genetic interpretation of the data. Some stains, such as esterases, phosphates,

and peroxidases, are not substrate specific and detect entire groups of enzymes. Isozymes that are coded by multiple loci are usually resolved by these stains, so banding patterns are often very complex and difficult to interpret. In addition, esterases, phosphatases, and other nonregulatory enzymes usually display more genetic variation than do regulatory enzymes involved with energy metabolism.<sup>16</sup> Utilization of only nonregulatory enzymes may detect a disproportionately high level of intraspecific variation and greatly overestimate the amount of genetic diversity in a population. On the other hand, the variability associated with nonregulatory enzymes may be desirable for identifying or “fingerprinting” subspecific taxa such as races or *formae speciales*.

#### D. GENETIC INTERPRETATION

Electrophoretic banding patterns of isozymes can be interpreted in terms of the alleles and loci that code for the polypeptides. Specific banding patterns are associated with certain genetic conditions, as described in Section II, and can be easily recognized. Journals often accept such genetic interpretations of the data without concurrent crossing experiments between isolates as long as the banding patterns are clear and always consistent with the genetic interpretation.

Electrophoretic data can be presented in many different forms. Statistical methods have been developed for population genetics, numerical taxonomy, and cladistics (i.e., systematic based on phylogenetic relationships) to express relatedness among samples. Each band on a gel can be assigned a descriptive value based either on the net migration of the band from the origin (an Rf value) or its position relative to that of the band coded by the most common allele.<sup>11</sup> Data can be analyzed using a variety of tests. If no genetic interpretation is planned, each pair of isolates can be compared using a variety of simple matching coefficients.<sup>17</sup>

More complex comparisons can be made when the data are interpreted in terms of loci and allele frequency. The data can be expressed in terms of genetic similarity (which describes the closeness of the relationship of two individuals or populations) or genetic distance (which indicates the amount of dissimilarity between two individuals or populations). Similarity values vary from 0 to 1.0 closely related organisms have values close to 1.0. Distance values vary from 0 to infinity; closely related organisms have distance coefficients close to 0. Different formulas can be used for calculating genetic distance and similarity.<sup>17-21</sup> Theoretical aspects of the use of some of these different statistical values are discussed by Buth.<sup>22</sup> Examples of calculations are presented by Ferguson.<sup>23</sup>

Matching, similarity, and distance coefficients can then be subjected to cluster analysis using multivariate analysis or other clustering procedures<sup>17</sup> to group together the different individuals or populations that resemble each other and to identify causes of variability (i.e., geographic location, subspecific groupings, etc.). Several cluster analysis programs are available that use slightly different parameters to analyze the data. Two of the most commonly used clustering procedures are single-linkage cluster analysis and unweighed pair group mean average cluster analysis.<sup>17,24</sup> More complex cladistic procedures can also be used.<sup>25</sup> The relationships of individual isolates or entire populations can be summarized in the form of clusters or dendrograms.<sup>23,26</sup> Most standard statistical software packages will perform the calculations necessary for isozyme analysis. More specialized programs are also available (e.g., “Allozyme,” R. Struss, University of Arizona, Tucson).

### IV. APPLICATIONS OF ISOZYME ANALYSIS

Many aspects of plant pathology, both applied and basic, can be studied with isozyme analysis.<sup>2,27</sup> Most applications have involved fungal pathogens, but the technique has also been used for nematodes<sup>28</sup> and bacteria.<sup>29,30</sup> Isozymes are frequently used by plant geneticists and breeders as genetic markers for resistance. This application is very important for plant pathology, but it is beyond the scope of this paper. Readers interested in using isozyme analysis to study host plants are referred to Conkle et al.,<sup>9</sup> Cheliak and Pitel,<sup>31</sup> Conkle,<sup>32</sup> and Tanksley and Orton.<sup>33</sup>

#### A. TAXONOMY

Isozyme analysis is frequently used for taxonomic purposes, especially when a taxon is morphologically diverse or plastic. In most cases, fungal species are easily differentiated by electrophoresis. The technique is commonly used to make recommendations on the separation or combination of species.<sup>34-39</sup> Subspecies, varieties, and intersterility groups have also been separated.<sup>40-44</sup> Simple band-counting procedures can be used to distinguish taxa, although cladistic and phylogenetic information can be derived from the

allelic frequencies and ratios derived from a genetic interpretation of the data. Isozyme analysis is most successful in distinguishing species and subspecies when the amount of intraspecific genetic variation is limited within a population. Otherwise, intraspecific variability will obscure interspecific differences. The selection of enzyme systems is very important in taxonomic applications (Section III, C). Any study that uses only nonregulatory enzymes or stains that visualize broad classes of enzymes (such as esterases, alkaline/acid phosphatases, and peroxidases) will display disproportionately high levels of intraspecific variation. Such a study would probably not be able to resolve taxonomic issues. Exaggerated levels of intraspecific variability can often be avoided by using both regulatory and nonregulatory enzymes and by using specific stains that react with single enzymes.

One important taxonomic question is how much variability can be allowed within a taxon before it should be split into a new species or subspecies. Thorpe<sup>45</sup> and Ayala<sup>46</sup> have provided guidelines for the separation of populations, subspecies, and species based on statistical interpretations of isozyme data derived from vertebrates, invertebrates, and plants. Such guidelines should be applied to fungi cautiously. Many genera, in which species are clearly defined, may fall into such discrete categories.<sup>47,48</sup> Other genera consist of poorly delineated species that exist in a continuum or “complex”. Using restrictive statistical cutoff values may eliminate outgroups that really belong within the continuum. Other fungal attributes, including differences or plasticity in morphology, cultural characteristics, and host preference, must be taken into account when deciding whether or not an organism needs to be reclassified.

## B. IDENTIFICATION OF UNKNOWN ORGANISMS

The ability of isozyme analysis to differentiate species and subspecies leads to its application in the identification of plant pathogens. Isozyme analysis can be used both to identify unknown pathogens and to “fingerprint” commercially important strains. The correct, rapid identification of an unknown pathogen may allow early implementation of control measures that will prevent large economic loss. State and federal agencies also need to be able to identify pathogens of regulatory significance, often from very small samples. Industry must be able to identify commercial strains that have been developed and patented. Of all the applications of isozyme analysis, pathogen identification is the one most important economically. This topic has been recently reviewed.<sup>1</sup>

The identification of unknown pathogens is dependent on the identification of monomorphic loci; i.e., loci that are invariable within a species (or subspecies). This must be determined by screening large numbers of isolates from a broad geographic range for many different enzyme systems and selecting those loci that do not demonstrate intraspecific (or intra-subspecific) variability. Subsequent electrophoretic runs should include a standardized strain of the suspected pathogen for comparison. Enzyme preparations of the standard strain can usually be prepared in large quantities and stored in liquid nitrogen for 1 year.

Isozyme analysis can also be used to identify the various pathogens present in a mixed infection. This has been especially useful in identifying mycorrhizal fungi.<sup>49,51</sup>

The “fingerprinting” of specific strains is dependent on the presence of polymorphic loci within the species. An allele, or combination of several alleles, must be identified that is unique to that particular strain. Often, enzymes that express high degrees of variability, such as esterases, phosphatases, and peroxidases, are useful for this application. Roux and Labarere,<sup>52</sup> for example, found that even closely related strains of *Agaricus bitorquis* (Quel.) Sacc. could be differentiated by their banding patterns for alcohol dehydrogenase, phenoloxidase, esterase, and peroxidase. Strains of commercial mushrooms,<sup>52,56</sup> biological control agents,<sup>57,59</sup> and mycorrhizal fungi<sup>60</sup> have been successfully “fingerprinted” with this technique. Isozyme analysis is easier and considerably less expensive than comparable molecular biological techniques that are used for strain identification, such as restriction fragment length polymorphism.

## C. GENETICS

Genetic information about a pathogen can be derived from isozyme analysis, including the amount of genetic variability (i.e., the percent polymorphism) of a species or population, the amount of heterozygosity, the linkage of specific loci, and genetic maps of the chromosomes. As genetic markers, isozymes are useful for studying population structure, tracing epidemics, establishing the origins of new pathogenic forms, and analyzing crosses.<sup>61</sup>

Isozymes and virulence are the most common markers used in fungal population genetics. Isozymes are generally more selectively neutral than virulence genes and usually demonstrate less variability.<sup>62,63</sup>

The inheritance of virulence may be quite complicated, involving dominance and recessiveness. The genetic patterns associated with isozymes are usually more simple; isozymes are usually expressed as codominant alleles at one locus or a few loci.<sup>64</sup> Virulence studies are also quite labor intensive, involving large numbers of different hosts, thus restricting sample size.<sup>65</sup> Isozyme tests can easily accommodate large numbers of samples.

The sample size, number of loci studied, and accuracy of species definition are all essential to obtaining valid estimates of genetic diversity. The type of enzyme selected is also important, since some enzymes are known to be more variable than others. Isozyme analyses that look only at esterases, phosphatases, and polyphenoloxidases, for example, would greatly overestimate the amount of variability in the genome.

The amount of genetic variability of a population has important implications for plant pathologists. Pathogens with a large amount of genetic diversity are more likely to become rapidly resistant to fungicides or virulent to resistant hosts. The amount of variability in a species (or population) is often related to the pathogenicity of an organism (Table 1). Obligate pathogens, which are highly specialized and have a relatively uniform substrate and environment often are very uniform genetically and have low levels of polymorphism, even for neutral markers.<sup>61,76</sup> *Erysiphe graminis* f. sp. *hordei*, for example is entirely monomorphic for over 50 different loci.<sup>88</sup> Certain hosts, such as barley, may impose strict biochemical requirements on pathogens and prevent the survival of recombinant forms.<sup>88</sup> Facultative pathogens and saprophytes, which find themselves in much more diverse environments and broader host ranges, are usually more genetically variable than obligate pathogens.<sup>61,76</sup> There are exceptions, however. For example, *Uromyces appendiculatus* (Pers.: Pers.) Unger, the causal agent of bean rust, is an obligate, autoecious, macrocyclic pathogen, yet 67% of its loci are polymorphic.<sup>66</sup> In contrast, *Fusarium oxysporum*, a pathogen with a very broad host range, displays only 24% polymorphism.<sup>47</sup>

The amount of polymorphism is also dependent on the amount of sexual reproduction in a population or species. Low levels of genetic diversity are often associated with species that are maintained asexually, such as *Phakopsora pachyrhizi* Sydow<sup>89</sup> and *Puccinia striiformis*.<sup>87</sup> There was no variation among *P. graminis* f. sp. *tritici* collections from Australia, where the fungus is maintained asexually.<sup>86</sup> Collections from the U.S., where sexual reproduction was common until the eradication of the barberry in the 1920s to 1930s, contain 38% polymorphic loci.<sup>78</sup> In contrast, sexual populations of *U. appendiculatus* displayed less genetic diversity than did asexual populations.<sup>66</sup> The authors concluded that mutation and selection would lead to greater divergence and higher levels of polymorphism in an asexual population since there is no exchange of genes. Fungi with very high reproductive potential, including those that form massive quantities of asexual spores, may possess tremendous genetic diversity due to mutation alone.<sup>70</sup> Lack of genetic variation may also indicate that a pathogen has developed from a limited number of recent introductions and that insufficient time has elapsed for variation to develop.<sup>70,82</sup> Low variability may also suggest that electrophoretic variants are unfit for survival or that coadapted isozyme complexes, which have similar electrophoretic migration rates, may exist.<sup>64</sup> Clearly, genetic diversity (or uniformity) can result from several different factors.

Genetic diversity can also be measured as the average number of alleles per locus or by percentage of heterozygosity in a population or species. Again, the selection of enzymes is very important (see Section III) since the loci must be representative of the genome. Heterozygous banding patterns are often readily recognizable (Section II). The frequency of heterozygous loci appears to be quite variable in fungi (Table 2). In most instances, estimates of heterozygosity are extremely conservative, since electrophoresis only detects one third of the heterozygosity that actually exists.<sup>3</sup> Overestimates of heterozygosity are a danger when working with heterokaryotic species, since the organism may be expressing the gene products of different homozygous nuclei rather than one heterozygous nucleus.<sup>82</sup>

Isozymes can also be used as markers to trace hybridizations that occur naturally or are induced, Burden et al.<sup>91</sup> used isozyme analysis to show that a common Australian race of *P. graminis* f. sp. *tritici* originated as a somatic hybrid of other races. Another study with *P. graminis* in Australia demonstrated that collections made from the grass *Agropyron scabrum* (Labill.) Beauv. originated as a somatic hybrid of *P. graminis* f. sp. *tritici* and *P. graminis* f. sp. *secalis*. Linde et al.<sup>63</sup> used phosphoglucomutase as a marker to examine selfing and crossing in the common bean rust fungus *U. appendiculatus*. They concluded that pathogens artificially increased in the greenhouse may not represent original populations in the field. Isozyme markers were also used to demonstrate that urediniospores could act as spermatia in isolates of *U. appendiculatus* that fail to initiate a sexual cycle.<sup>92</sup>

**Table 1 Polymorphic loci in fungal populations and species as estimated by isozyme analysis**

Species	Polymorphism (%)	Ref.
<i>Pleurotus ostreatus</i> (Jacq. ex Fr.) Quel.	94	66
<i>Agaricus campestris</i> Fr.	87	67
<i>Atkinsonella hypoxylon</i> (Peck) Diehl	85	48
<i>Suillus variegatus</i> (Fr.) O. Kuntze	71	50, 51
<i>S. plorans</i> (Roll.) Sing.	67	60
<i>Uromyces appendiculatus</i> (Pers.) Unger	67	68
<i>Puccinia graminis</i> Pers. f. sp. <i>tritici</i> (worldwide)	61	69
<i>Pyricularia oryzae</i> Cavara	55	70
<i>Lentinula edodes</i> (Berk.) Pegler	55	71
<i>Phytophthora infestans</i> (Mont.) deBary	54	72
<i>Tilletia indica</i> Mitra	44, 52	73, 74
<i>S. bovinus</i> (Fr.) O. Kuntze	50	50, 51
<i>S. tomentosus</i> (Kaufm.) Snell, Singer & Dick	47	50, 51
<i>S. placidus</i> (Bon.) Sing.	47	60
<i>Agaricus brunnescens</i> Peck	43	54
<i>Heterobasidion annosum</i> (Fr.) Bref. Intersterility group "spruce"	40	75
<i>Ustilago zaeae</i> (Beckm.) Ung.	40	76
<i>Rhizoctonia solani</i> Kuehn	38	77
<i>Rhynchosporium secalis</i> (Oudem.) J. J. Davis	38	76
<i>Cryphonectria cubensis</i> (Bruner) Hodges	38	37
<i>Puccinia graminis</i> f. sp. <i>tritici</i> (U.S.)	38	78
<i>Endocronartium harknessii</i> (J. P. Moore) Y. Hiratsuka	38, 12	79, 80
<i>Ceratocystiopsis ranunculosis</i> J. R. Bridges & T. J. Perry	36	36
<i>Leptographium wageneri</i> (Kendrick) Wingfield	30, 48	42, 75
<i>Volvariella volvacea</i> (Bull. ex Fr.) Sing.	29	81
<i>Cronartium quercuum</i> (Berk.) Miyabe ex Shirai f. sp. <i>banksianae</i>	25	80
<i>Fusarium oxysporum</i> Schlect, emend Snyder & Hans.	24	47
<i>Peronosclerospora sorghi</i> (Weston & Uppal) C. G. Shaw	23	34
<i>Phytophthora cinnamoni</i> Rands	23	82
<i>Heterobasidion annosum</i> Intersterility group "pine"	20	43, 75
<i>Erysiphe graminis</i> D.C. f. sp. <i>tritici</i>	20	83
<i>E. graminis</i> f. sp. <i>secalis</i>	20	83
<i>Stagonospora nodorum</i> (Berk.) Castellano & E. G. Germano	11	76
<i>U. spinificis</i> Ludw.	11	84
<i>Pyricularia oryzae</i> (rice isolates)	11	70
<i>U. bullata</i> Berk.	11	85
<i>Puccinia recondita</i> Robs. ex Desm. f. sp. <i>tritici</i>	9	86
<i>P. striiformis</i> West. f. sp. <i>tritici</i>	0	87
<i>P. striiformis</i> f. sp. <i>hordei</i>	0	87
<i>P. graminis</i> f. sp. <i>tritici</i>	0	86
<i>P. hordei</i> Otth.	0	87
<i>E. graminis</i> f. sp. <i>hordei</i>	0	88

Careful analysis of isozyme patterns can be used to determine whether individual loci are inherited independently or are linked. This information can be used to form genetic maps of chromosomes. Nine different loci were inherited independently in *U. appendiculatus*; there was no evidence for linkage.<sup>64</sup> Two of six genetic loci were linked in *Lentinula edodes*.<sup>71</sup> The frequency of crossing over was used to estimate the distance of two linked loci from the centromere in *Agaricus brunnescens*.<sup>93</sup> Similar linkage studies have been done with *A. campestris*,<sup>65</sup> *Volvariella volvacea*,<sup>81</sup> *P. graminis* f. sp. *tritici*,<sup>69</sup> and *Ustilago bullata*.<sup>94</sup>

**Table 2 Heterozygosity in different fungal species as estimated by isozyme analysis**

Species	Heterozygosity (%)	Ref.
<i>Tilletia indica</i> Mitra	7.2	73
<i>Ustilago bullata</i> Berk.	4.8	85
<i>Puccinia graminis</i> Pers. f. sp. <i>tritici</i>	33.0	86
<i>P. recondita</i> Robs. ex Desm. f. sp. <i>tritici</i>	15.0	86
<i>Agaricus campestris</i> Fr.	28.0	65
<i>Scytinostroma galactinum</i> (Fr.) Donk	0.0	90
<i>S. protrusum</i> (Burt)		
Nakas. subsp. <i>protrusum</i> Nakas.	12.5	90
<i>S. protrusum</i>		
subsp. <i>septentrionale</i> Nakas.	37.5	90
<i>Heterobasidium annosum</i> (Fr.) Bref. intersterility group S	10.0	43
<i>H. annosum</i> intersterility group P	2.0	43

The ploidy level (haploid, diploid or dikaryotic, and polyploid) of a fungus can often be determined from isozyme data (Section II). Studies of the life cycle of the organism can thus be performed. Isozyme analysis was used to show that mating patterns of *Phytophthora infestans* were random in Mexico, where the sexual stage of the pathogen exists.<sup>72</sup> Asexual populations were identified by lack of recombination in the U. S., Canada, and Europe. In England and Wales, 10% of *P. infestans* isolates were of the uncommon A2 mating type (usually found only in Mexico).<sup>95</sup> Additional circumstantial evidence attested to low levels of sexual reproduction in the U.K.<sup>95</sup> Low levels of recombination were also demonstrated in *Rhynchosporium secalis*, an organism that has no known sexual phase.<sup>65</sup>

Atypical meiosis has been detected in some organisms by isozyme analysis. Basidiospores arising from germinated teliospores of *Tilletia indica* did not inherit alleles with equal frequency, and some basidiospores appeared to inherit both alleles.<sup>35</sup> The authors proposed that some basidiospores may receive two haploid nuclei from the promycelium or that the spores are actually aneuploids. This interpretation has been supported by cytological evidence.<sup>96</sup> Atypical meiosis has also been demonstrated in homokaryotic lines of *A. brunnescens*.<sup>93</sup>

#### D. EPIDEMIOLOGY

In many cases, fungi of a single species from different geographic sources can be differentiated from each other by isozyme analysis. This usually occurs due to genetic isolation. The differentiation of electrophoretic patterns in isozyme analysis is often the first indication that organisms are beginning to evolve into different species. Such information can be used to identify the origin of pathogens and to document their movement.

Cluster analysis was used to place isolates of *Endocronartium harknessii*, causal agent of western gall rust, into two distinct groups that corresponded to their geographic source.<sup>79</sup> This confined an earlier premise that *E. harknessii* is a western rust and that a different species occurs in the eastern U.S. Local populations of *Morchella deliciosa* Fr., *M. esculenta* (L.) Pers.,<sup>97</sup> *Neurospora intermedia* Tai,<sup>98</sup> and *Suillus* spp.<sup>50,51,60</sup> were also shown to be genetically distinct. Only 7% of alleles were shared among isolates of *Phakopsora pachyrhizi* from the Eastern and Western hemispheres.<sup>35</sup> When genetic distances are so large, the original classification of the organisms as a single species must be questioned. Isozyme patterns have been used to trace independent introductions of *Puccinia recondita* Robs. ex Desm. to the U.S.<sup>99</sup> and *P. graminis* f. sp. *tritici* to Australia.<sup>100</sup>

Geographic localities in which large amounts of genetic variability are encountered often represent the place of origin of a species. Kerrigan and Ross<sup>101</sup> thus speculated that *A. bisporus* (Lange) Imbach is indigenous to North America, just as Tooley et al.<sup>72,102</sup> traced the evolutionary origin of *Phytophthora infestans* to Mexico.

#### E. PATHOGENICITY AND VIRULENCE

There have been mixed results in using isozyme analysis to differentiate races, *formae speciales*, and other subgroups differing in host preference, pathogenicity, and virulence. Such separations have been made for some fungi, including *Cronartium quercuum*,<sup>103</sup> *F. oxysporum*,<sup>47,104</sup> *Erysiphe graminis*,<sup>83</sup> *Hetero-*

*basidion annosum*,<sup>43</sup> *Atkinsonella hypoxylon*,<sup>48</sup> *Cochliobolus carbonum* R. R. Nels.,<sup>105</sup> *Cryphonectria cubensis*,<sup>106</sup> *Phytophthora megasperma* Drechs.,<sup>107</sup> *Puccinia graminis*,<sup>87</sup> *P. sorghi* Schwein.,<sup>108</sup> and *Phyllostopsis nidulans* (Pers. ex Fr.) Sing. [= *Pleurotus nebrodensis* (Pers. ex Fr.) Kummer].<sup>109</sup> Races of *P. graminis* f. sp. *tritici* have been separated, but only in asexual populations.<sup>78</sup> Sexual populations of the organism had much higher levels of genetic variability, which obscured differences associated with virulence. In many cases, the genes responsible for virulence are quite distinct and are different from those assayed in isozyme analysis. There is usually more genetic variability among virulence genes than is detected by isozyme analysis due to the strong selection pressures placed upon pathogens to infect and colonize resistant hosts.

## V. ADVANTAGES AND DISADVANTAGES

Isozyme analysis, as any technique, has its strengths and weaknesses. With starch gel electrophoresis, the technique is relatively inexpensive and results in less exposure to toxic chemicals (except for certain stain components). More staining systems can be used with starch gel electrophoresis than PAGE. This allows the researcher to compare large numbers of enzymes from many different metabolic pathways and to obtain information about many different genetic loci. In most cases with fungal pathogens, good resolution and successful genetic interpretations can be obtained with a panel of 15 to 25 enzymes. Such an isozyme test can be completed within a day. Most stains used in isozyme analysis are specific for a single enzyme. This greatly simplifies data interpretation since only a limited number of bands are visualized for each sample. In contrast, general protein stains usually detect large numbers of bands, which makes data collection and interpretation (e.g., the calculation of simple matching coefficients or other statistical values) very difficult.

The greatest disadvantage to isozyme analysis is the relatively large quantities of an organism that are often required for extracting sufficient enzyme. This is usually not a problem with facultative fungi that can be cultured on artificial media. Obligate pathogens may require considerable effort to obtain the necessary quantities of fungal tissue. For example, with maize downy mildew fungi, conidia can be washed directly from the plant surface and concentrated by centrifugation.<sup>34,38</sup> Similar difficulties arise when analyzing urediniospores of rust fungi. In general, 50 to 100 mg (wet weight) of mycelium, 50 mg (wet weight) of downy mildew conidia, or 30 to 50 mg (pregermination dry weight) of germinating rust urediniospores are needed for isozyme analysis.

Time requirements may be another disadvantage to isozyme analysis, depending upon the application. Although electrophoresis can be conducted rapidly, several days or even weeks are often needed to isolate and grow the organisms. For example, the germination of viable teliospores of *T. controversa* Kuhn, the causal agent of dwarf bunt of wheat, can require 6 to 8 weeks, and continued growth of mycelium is then necessary for another week to obtain sufficient fungal material for the test.<sup>1</sup> Such time requirements are unacceptable in situations where identification is required within hours. In such cases, alternative procedures, such as gene probes, may be more satisfactory.

Using isozyme analysis with bacteria presents another problem. The slimy polysaccharides of the capsule must be removed, using ultracentrifugation or enzymatic treatments.<sup>1,29</sup> Even without excess carbohydrate, the electrophoretic migration rates of bacterial proteins are often quite similar, and only small differences in banding patterns may be observed. It is usually necessary to repeat the analysis several times to ensure that the bands are scored correctly. Alternative methods, including various nucleic acid procedures, may be more effective in separating and analyzing bacterial plant pathogens.

## VI. CONCLUSIONS

Isozyme analysis is a simple, efficient, and inexpensive technique for evaluating the taxonomy, genetics, virulence, and epidemiology of plant pathogens, especially fungi. The technique also has practical applications for pathogen detection and identification. Recently, there has been an explosion in the number of publications describing the application of isozyme analysis to phytopathological questions. Objections raised by "classical" geneticists have subsided as genetic interpretations of banding patterns have been confirmed by crossing experiments. Isozyme analysis is becoming a standard technique for the study of plant pathogens.

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