Isozyme analysis of Paecilomyces farinosus and Paecilomyces fumosoroseus (Deuteromycotina: Hypomycetes), two potential biological control agents of the sweet potato and silverleaf whiteflies (Bemisia spp.)

by Joseph E Bunnell

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Entomology
Montana State University

Abstract:
The sweet potato whitefly, Bemisia tabaci Gennadius, and silverleaf whitefly, Bemisia argentifolii Bellows and Perring, are two economically important pests of crops that together cause an estimated three quarters of a billion dollars damage annually. Two fungal pathogens, Paecilomyces farinosus (Holm ex Gray) Brown and Smith, and Paecilomyces Jumosoroseus (Wize) Brown and Smith, are being investigated as to their potential for controlling the two whitefly species. Currently, the literature is bereft of molecular markers for Paecilomyces spp., unlike the case with other fungal biological control agents such as Beauveria spp. and Metarhizium spp. Twenty-three isolates of P. farinosus and P. fumosoroseus were selected to generate isozyme profiles which would aid in identification at the species level. The estimated genetic variability at the intraspecific level was quantified for these twenty-three isolates. Thirty-four enzyme-buffer systems were used in the screening run. Of those, twelve proved useful to consistently and reproducibly distinguish between the two species. Nine consistently banding enzyme-buffer systems showed no polymorphisms among all isolates. Mean genetic distances ranged from 0.0617 (PFR603) to 0.2069 (PF601). Cluster analysis showed one tight group (mostly P. fumosoroseus), and another loose group (mostly P. farinosus). Principle components analysis and nonmetric multidimensional scaling produced results in agreement with the cluster analysis.

ISOZYME ANALYSIS of *Paecilomyces farinosus* and *Paecilomyces fumosoroseus* (Deuteromycotina: Hyphomycetes), TWO POTENTIAL BIOLOGICAL CONTROL AGENTS of the SWEET POTATO and SILVERLEAF WHITEFLIES (*Bemisia* spp.)

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A thesis submitted in partial fulfillment
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of

Master of Science

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Entomology

MONTANA STATE UNIVERSITY
Bozeman, Montana

April 1995

APPROVAL

of a thesis submitted by

Joseph E. Bunnell

This thesis has been read by each member of the graduate committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Date 05 APRIL 1995

ACKNOWLEDGEMENTS

I offer my most heartfelt and sincere thanks to Dr. Robert M. Nowierski, who advised me in matters both academic and personal. I cannot overstate the degree to which his openness, encouragement, and inspiring motivation have helped me during my three years at M.S.U.

My committee members, Drs. William P. Kemp, Stefan T. Jaronski, and Luther E. Talbert, provided me with valuable comments and constructive criticism all throughout my thesis project. Each in his own way, they gave of themselves by sharing with me ideas, suggestions, and hard questions. Mr. Zheng Zeng freely and enthusiastically lent his expertise to the statistical analysis and my interpretion of those results.

While I cannot mention everyone by name to whom I owe my gratitude, I thank the following people: the lab crew, Jorge M. Brito, Bryan C. FitzGerald, Robert T. Grubb, and Steven Kearing; the office staff, Rose Adams, Joan Scarff, and Nancy Taylor; the individuals at Mycotech Corp. who grew the fungal cultures; the many scientists at M.S.U. and other universities for informal discussions, including William C. Black IV, Matt Lavin, Jack Martin, and Leonard E. Munsterman.

This project owes its existence and completion to the support of my family and friends, especially Bettina Borgstedt, Julie K. Bunnell, Leslie B. Cummings, Pablo Lusnia, Belinda Thom, my fellow graduate students; and by the grace of God.

TABLE OF CONTENTS

TABLE OF CONTENTS -- Continued

LIST OF TABLES

LIST OF FIGURES

GLOSSARY OF TERMS AND ABBREVIATIONS

ADP: adenosine diphosphate; Sigma A-6521 (formula wt. 427.2).

allele: one of several forms of the same **gene**, usually recognized by their phenotypic effects; they are believed to differ by mutation of the **DNA** sequence.

allozyme: one of several forms of an enzyme coded for by different **alleles** at a **locus**.

amerospored: (see **coenocytic**).

anastomosis: fusion between **hyphal** elements, forming a bridge.

AP-PCR: arbitrarily primed **PCR**; **RAPD-PCR**.

ATP: adenosine 5'-triphosphate; Sigma A-5394 (formula wt. 551.1).

assimilative: growing; food absorbing; growth prior to reproduction.

biological control: the suppression of a host or prey species by its natural enemies.

blastospore: spore that arises by budding.

caducous: readily deciduous.

coenocytic: possessing no **septa**.

conidiophore: structure which holds spores up or away from the assimilative **mycelium**.

conidium: a specialized, non-motile, asexual propagule, usually **caducous**, not developing by cytoplasmic cleavage or free-cell formation; asexual spore; **blastospore**; thin-walled secondary spore borne terminally upon a specialized **hypha** or **conidiophore**; uninucleate exogenous spore.

coremium: an erect, compact cluster of **conidiophores** (coremium may be a more definite form than **synnema**).

DNA: deoxyribonucleic acid; consisting of pairs of the bases adenine and thymine (A-T), and guanine and cytosine (G-C), held together by hydrogen bonding to form a double helix.

E.C.: enzyme committee; standardized enzymatic nomenclature according to the 1984 Nomenclature Committee of the International Union of Biochemistry.

EDTA: ethylenediaminetetraacetic acid (formula wt. 372.2)

eigenvalue: variance accounted for by a particular axis (component) in **PCA**.

electromorph: electrophoretic **phenotype**; zymogram.

enteroblastic: a mode of blastic conidium ontogeny in which the outer layer(s) of the wall of the conidiogenous cell is (are) not involved in the formation of the **conidium** wall.

epistasis: gene interaction.

exogenous: arising on the outside.

fungi: (plural of fungus, from Latin meaning "fungus, mushroom") a kingdom of parasitic (symbiotic) or saprophytic (decomposing) organisms.

gene: functional unit of heredity.

genetic distance: extent of genomic differences between **OTU**s that is measured by some numerical quantity.

heterokaryosis: condition of being multinucleate.

hyaline: transluscent, glassy, colorless.

hypha: fungal filament, of the assimilative or fruit body.

isozyme (*isoenzyme*): one of several forms of an enzyme, produced by different, nonallelic **loci** in an individual organism's genome; products of different **genes** sharing a common ancestor (divergent **phenotypes**).

linkage disequilibrium: nonrandom association of genes between different loci.

locus (pl. *loci*): site on a chromosome occupied by a specific **gene**; the gene complex, in all its allelic states.

M: molar concentration (moles per liter).

Mbp: million base pairs (in **DNA**, A-T and G-C pairs).

mM: millimolar concentration (thousandth of a mole per liter).

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; thyazolyl blue; Sigma M-2128 (formula wt. 414.3).

mycelium: vegetative or assimilative stage of fungus; made up of septate **hyphae**, cylindrical filaments with walls enclosing (usually) multinucleate protoplasm; **thallus**.

NAD: β-Nicotinamide adenine dinucleotide; Sigma N-7004 (formula wt. 663.4).

NADH: β-Nicotinamide adenine dinucleotide, reduced form; Sigma N-8129 (formula wt. 709.4)

NADP: β-Nicotinamide adenine dinucleotide phosphate; Sigma N-0505 (formula wt. 765.4).

NADPH: β-Nicotinamide adenine dinucleotide phosphate, reduced form; Sigma N-7505 (formula wt. 833.4)

NMDS: nonmetric multi-dimensional scaling.

NPV: nuclear polyhedrosis virus.

OTU: operational (operative) taxonomic unit; *e.g.*, species, isolate, population.

PCA: principle components analysis; a linear ordination method of multivariate statistics, represented graphically in a reduced coordinate system.

PCR: polymerase chain reaction.

PF: *Paecilomyces farinosus*.

PFR: *Paecilomyces fumosoroseus*.

PGI: Phosphoglucose isomerase; Sigma P-9010 (D-Glucose-6-phosphate ketol-isomerase, E. C. 5.3.1.9).

phenotype: morphological, biochemical, behavioral, physiological, and other properties of an organism, manifested throughout its life, that develop through action of **genes** and environment; or any subset of such properties, especially those affected by a particular **allele** or other portion of the genotype.

phialide: a conidiogenous cell which produces, from a fixed conidiogenous **locus**, a basipetal succession of **enteroblastic** conidia whose walls arise *de novo*; an end cell of a **conidiophore**.

PMS: phenazine methosulfate (N-Methyldibenzopyrazine methyl sulfate salt); Sigma P-9625 (formula wt. 306.3).

PVP: polyvinylpyrrolidone (Sigma PVP-40).

RAPD-PCR: randomly amplified polymorphic **DNA - PCR**; **AP-PCR**.

R*f*: relative migration distance of a sample protein (enzyme) through a gel matrix as a result of electrophoresis compared to a reference with R*f* = 1.0.

RFLP: restriction fragment length polymorphism.

septum (pl. *septa*): cross wall; disc with pore in the middle, through which genetic material may flow between cells.

synnema: an erect, compact cluster of **conidiophores** (synnema may be less definite form than **coremium**).

thallus: 1. (fungi) the entire assimilative phase of the individual; 2. (general) vegetative portion of a non-vascular plant.

Tris: trizma base; Tris(hydroxymethyl)aminomethane; $C_4H_{11}NO_3$ (formula wt. 121.1)

verticel: whorl of spores.

ABSTRACT

The sweet potato whitefly, *Bemisia tabaci* Gennadius, and silverleaf whitefly, *Bemisia argentifolii* Bellows and Perring, are two economically important pests of crops that together cause an estimated three quarters of a billion dollars damage annually. Two fungal pathogens, *Paecilomyces farinosus* (Holm ex Gray) Brown and Smith, and *Paecilomyces fumosoroseus* (Wize) Brown and Smith, are being investigated as to their potential for controlling the two whitefly species. Currently, the literature is bereft of molecular markers for *Paecilomyces* spp., unlike the case with other fungal biological control agents such as *Beauveria* spp. and *Metarhizium* spp. Twenty-three isolates of *P. farinosus* and *P. fumosoroseus* were selected to generate isozyme profiles which would aid in identification at the species level. The estimated genetic variability at the intraspecific level was quantified for these twenty-three isolates. Thirty-four enzyme-buffer systems were used in the screening run. Of those, twelve proved useful to consistently and reproducibly distinguish between the two species. Nine consistently banding enzyme-buffer systems showed no polymorphisms among all isolates. Mean genetic distances ranged from 0.0617 (PFR603) to 0.2069 (PF601). Cluster analysis showed one tight group (mostly *P. fumosoroseus*), and another loose group (mostly *P. farinosus*). Principle components analysis and nonmetric multidimensional scaling produced results in agreement with the cluster analysis.

1. INTRODUCTION

The whitefly family Aleyrodidae contains approximately 1200 species, including a few economically important species such as *Trialeurodes vaporariorum* Westwood, which is a serious pest of many plant species grown in glasshouses, and a few *Bemisia* spp. which are economically damaging pests of crops and ornamentals (Byrne *et al.* 1990). Whiteflies secrete a sticky substance called "honeydew" (comprised partly of trehalulose and other sugars), which interrupts harvesting machinery and milling processes, particularly in cotton (Becker *et al.* 1992), provides a vehicle for viral, fungal, and other disease transmission (Gill 1992), and reduces photosynthetic efficiency by elevating leaf temperatures (Byrne *et al.* 1990).

The sweet potato whitefly, *Bemisia tabaci* Gennadius, which is most likely a species introduced into North America, has a wide host plant range (Becker *et al.* 1992), including at least 500 plant species (Dowell 1990) in 18 families (Gill 1992). It causes approximately three quarters of a billion dollars damage annually. *B. tabaci* has acquired resistance to many insecticides, and is difficult to control effectively with chemicals because of its preference for the undersides of leaves. For these reasons, as well as environmental concerns, development of biological control efforts as part of an Integrated Pest Management (IPM) program is imperative.

Another *Bemisia* species, *B. argentifolii* Bellows and Perring, which is called the silverleaf whitefly, is also a serious pest of crops and ornamentals. The description of this species of whitefly was based on collections made in California and

Florida (Bellows *et al.* 1994). The economic importance of *B. argentifolii* must be substantial, although previous damage estimates have been obscured by the failure to distinguish this species from *B. tabaci* prior to 1994. Currently, it is thought that *B. argentifolii* displaced *B. tabaci* in the mid 1980s, which explains why what was once thought to be a single species of whitefly went from being a relatively minor pest to the very serious one that it is today (Jaronski 1995, pers. comm.). Biological differences between the two species include *B. argentifolii*'s larger size, higher rate of honeydew production, host range, greater fertility and fecundity (Bellows *et al.* 1994).

The entomopathogenic fungi *Paecilomyces farinosus* (Holm ex Gray) Brown and Smith and *P. fumosoroseus* (Wize) Brown and Smith are currently being developed as potential biological control agents of *Bemisia tabaci* and *B. argentifolii*. Of central importance to the effective use of microbial insecticides is the assurance of proper identification (see *Paecilomyces* spp., Identification, p 8) . Descriptive keys presently rely predominately on morphological characters to distinguish among fungal species and isolates. The use of such characteristics has proven highly ambiguous, especially for *P. farinosus* and *P. fumosoroseus* (Jaronski 1993, pers. comm.).

This thesis represents part of an effort to use and refine a taxonomic approach that integrates other criteria, specifically the biochemical tool of enzyme electrophoresis (*i.e.*, isozyme/allozyme analysis). Other data which are typically used to provide a more accurate description of taxa, such as the results of breeding, or crossing experiments, are unobtainable in the case of *Paecilomyces* spp. due to their asexual nature.

Objectives and Hypotheses

The objectives of this study were to distinguish between species and among selected isolates of *Paecilomyces* spp. by the use of isozyme analysis, and to estimate genetic variability among isolates using multivariate statistical methods based on Nei's genetic distances (Nei 1972).

The hypotheses are that molecular markers do exist that enable more reliable identification of these *Paecilomyces* spp. than are presently available using morphometric characters, and that the segregation of loci which code for individual enzymes is nonrandom.

2. LITERATURE REVIEW

Bemisia spp.

Due to the very recent recognition of *B. argentifolii* in the literature, much of the information that follows will have to be presumed to include both species under the misnomer of a single name of *B. tabaci*.

Range and Ecology

While it is generally accepted that *B. tabaci* has as its geographic origin Africa, Asia, or the Middle East, Gill (1992) suggests a New World origin. Today, its U.S. range encompasses Arizona, California, Florida, Georgia, Hawaii, and Texas. This whitefly is now found in Greece (the location of its original description in 1889), northwestern Mexico, Australia, Brazil, China, Egypt, Fiji, India, Iran, Israel, Italy, Japan, Madagascar, Malaysia, New Guinea, Nigeria, Nicaragua, Pakistan, the Philippines, Russia, Spain, Sri Lanka, the Sudan, Taiwan, Thailand, Turkey, Venezuela, the West Indies, Zäire, Zimbabwe, much of the remaining African and southern European countries, and the Middle East (Gill 1992; Jaronski 1995, pers. comm.). Although *B. tabaci* was first reported in the United States in the 1920s (in Florida), it has probably been here under a pseudonym since the 1900s (Gill 1992).

B. tabaci usually deposits spindle-shaped eggs on the undersides of leaves, especially those of new growth (Simmons 1994). The first instars crawl around the leaf surface in search of a suitable feeding location, while the remaining three instars are essentially sedentary scales (Osborne and Landa 1992). The whitefly immatures then enter a pupal stage and later emerge as winged adults (Gill 1992). Upon emergence, adults' wings are glabrous and hyaline to white in color. The life cycle is completed in 21 to 25 days, depending on temperature.

Economic Importance

B. tabaci is a major pest of such crops as sweet potato, cotton, lettuce, tomato, soybean, cucurbits (Osborne and Landa 1992), alfalfa (Becker *et al.* 1992), cassava, citrus, melons, okra, soy bean, squash, sugar beets, tobacco (Gill 1992); as well as such ornamentals as pointsettias and *Hybiscus* spp. (Martens 1993). It caused an estimated half billion dollars damage in 1991 (Bellows, Jr. *et al.* 1994), and $750 million in 1992 (Jaronski 1993, pers. comm.). Damage by the insect to the crops is of two types: direct feeding damage, and secondary damage following the deposition of copious amounts of honeydew. The honeydew produced by this whitefly provides a substrate for black sooty molds which often cause reduced photosynthesis, sunburning, and decreased yields in affected plants (Byrne *et al.* 1990). Honeydew also causes sticky cotton which, due to problems in ginning, is unmarketable or brings very reduced prices to the producer.

B. tabaci is the main whitefly vector of viruses such as bean golden mosaic virus (BGMV), African cassava mosaic virus (ACMV), lettuce infectious yellow virus (LIYV) (Byrne *et al.* 1990), tomato yellow leaf curl geminivirus (TYLCV) (Navot *et al.* 1992), and squash yellow leaf curl virus (SYLCV) (Gill 1992). *B. tabaci* also has acquired resistance to many synthetic insecticides (Osborne and Landa 1992).

Control Strategies

Many commonly used insecticides, including mecarbam, aldicarb, methyl parathion, amitraz, dimethoate, monocrotophos, and such pyrethroids as cypermethrin, deltamethrin and cyhalothrin, can be effective in controlling outbreaks (Dittrich *et al.* 1990). However, the long term management of this whitefly will be difficult due to the widespread resistance to insecticides shown by this whitefly species (Dowell 1990; Osborne and Landa 1992). Resistance to newer chemicals, such as imidocloprid and IGR buprofezin, has already been demonstrated (Jaronski 1995, pers. comm.).

The list of endemic and introduced natural enemies of *B. tabaci* numbers more than fifty-five species, including such fungal pathogens as *Paecilomyces* spp., *Beauveria bassiana* (Balsamo) Vuillemin (Fransen 1990; Onillon 1990; Becker *et al.* 1992), *Aschersonia aleyrodis* Webber, *Verticillium lecanii* (Zimmerman) Viegas (Osborne and Landa 1992); the parasitoids: *Trichogramma chilonis* Ishii (Dhandapani *et al.* 1992), *Eretmocerus californicus* Howard, *Er. mundus* Mercet, *Encarsia formosa*

Gahan, *En. nigricephala* Dozier, *En. transvena* (=*sublutea*) Timberlake, and *En. tabacivora* Viggiani (Becker *et al.* 1992); and the predators: big-eyed bug, *Geocoris punctipes* (Say) (A. C. Cohen 1994, pers. comm.), *Brinckochrysa (Chrysopa) scelestes* Banks, and *Delphastus pusillus* LeConte (Dhandapani *et al.* 1992).

Evidence for creation of a new species ("silverleaf whitefly," "pointsettia strain," "biotype B")

The following scientific criteria have led to the recognition of a new species of whitefly, known as *Bemisia argentifolii*: Lack of interbreeding (biological species definition), RAPD-PCR (AP-PCR) evidence (DNA polymorphisms) (Perring *et al.* 1993), presence of sugar, "bemisiose," not previously described in nature (Becker *et al.* 1992), morphological differences and isozyme analysis (Bellows, Jr. *et al.* 1994).

B. argentifolii differs from *B. tabaci* in that it is more cold tolerant, completes its life cycle in a shorter time (16 to 23 days), and is estimated to be five times more prolific (Gill 1992). Crops which *B. argentifolii* attacks, in addition to those listed above for *B. tabaci*, include broccoli and table grapes (Gill 1992). The transmission of the disease, "squash silver leaf," (probably the response to a phytotoxin) led to *B. argentifolii*'s common name, silverleaf whitefly (Gill 1992).

Paecilomyces spp.

Members of the genus *Paecilomyces* are commonly found in nature and to date include 31 described species. A number of these fungal species are entomopathogenic, including *P. farinosus*, *P. fumosoroseus*, *P. amoeneroseus* (Hennings) Samson, *P. javanicus* (Friederichs and Bally) Brown and Smith, *P. ramosus* Samson and Evans, *P. coleopterorum* Samson and Evans, *P. tenuipes* (Peck) Samson, *P. cicadae* (Miquel) Samson, *P. lilacinus* (Thom) Samson, and *P. cinnamomeus* (Petch) Samson and Gams (Tanada and Kaya 1993).

Members of the genus *Paecilomyces* are homothallic (*i.e.*, monoecious), heterokaryotic, amerospored (coenocytic), phialidic (possessing hyaline conidiogenous hyphae), with verticels more or less flask-shaped (Griffin 1994), and have a coremium present. Conidiophores are long, tubular, bent away from the conidial bearing structures, and are not always in verticels (Hazen *et al.* 1970). The distribution of this genus is worldwide (Starnes *et al.* 1993).

Identification

Fungal identification has previously separated species and isolates on the basis of colony color and conidial size and shape (Onions 1979). As mentioned previously, the use of such morphological characteristics has proven highly ambiguous, especially for *P. farinosus* and *P. fumosoroseus* (Jaronski 1993, pers. comm.). For example,

P. fumosoroseus isolate PFR600A has been identified as *P. farinosus* by the U.S.D.A., due to phenotypic instability; *viz.* color and sporulating ability (Jaronski 1995, pers. comm.).

Reasons for insuring a highly reliable method for distinguishing among entomopathogenic fungal biological control agents include such concerns as differences in efficacy of various isolates within a species, release and redistribution of approved isolates only, quarantine, other governmental regulatory issues (Micales et al. 1986), and protection and maintenance of patentable lines (*e.g.*, Martens 1993). Moreover, as Roberts and Yendol (1971) point out, a single fungal species may contain strains which are highly divergent in virulence and physiology. The significance of such differences in fungal populations is one of evolutionary biology, not merely a concern with classification (Bidochka 1994).

There are certain inherent difficulties in fungal identification due to such unique phenomena as hyphal fusion and asexual propagation of spores. Burnett (1968) discusses the inherent confusion in even defining a fungal species, population, or individual. He concludes that a given mycelium in its natural environment is a genetic mosaic, while acting as a single ecological and physiological unit.

Paecilomyces farinosus

Paecilomyces farinosus, was originally described as *Spicaria farinosa* (Holm ex Gray) Vuillemin (Aizawa 1971; Roberts and Yendol 1971) and *Isaria farinosa*

(Holm ex Gray) Fries (Tanada and Kaya 1993), and has been recorded on a wide variety of hosts (Homoptera, Lepidoptera, Diptera, Coleoptera, Hymenoptera, and Arachnida). It has been investigated as a potential biological control agent of the codling moth, *Cydia pomonella* (Linnaeus); Colorado potato beetle, *Leptinotarsa decemlineata* (Say); *Heliothis armigera* (Hübner); grape phylloxera, *Daktulospharia vitifoliae* (Fitch); European pine shoot moth, *Rhyacionia buoliana* (Denis and Schiffermüller); gypsy moth, *Lymantria (=Porthetria) dispar* (Linnaeus) (Onions 1979); aphids (Hayden *et al.* 1992); and the migratory grasshopper, *Melanoplus sanguinipes* (Fabricius) (Khachatourians 1992).

The infection caused by *P. farinosus* is also known as yellow muscardine (Tanada and Kaya 1993). This species has been claimed as the imperfect stage of *Cordyceps memorabilis* Cesati (Pacioni and Frizzi 1978), and *C. militaris* (Link: Fries) Link, but Tanada and Kaya (1993) disagree.

Paecilomyces fumosoroseus

Insect hosts for *P. fumosoroseus* are found in the orders Homoptera, Lepidoptera, Diptera, Hymenoptera, Isoptera, and others (Onions 1979). The first documented use by *Paecilomyces fumosoroseus* for pest control was against peach fruit moth in 1959 (Onions 1979). Patented isolates (patent owner: University of Florida; license holder: W. C. Grace Co.) of *P. fumosoroseus*, originally isolated from naturally infected mealybugs, have been used successfully against *Bemisia tabaci*

(Osborne and Landa 1992; Martens 1993), spider mites, thrips, and aphids (Martens 1993). *P. fumosoroseus* has also been used in the control of the silkworm tachina fly, *Blepharipa zebina* (Walker), the peach pyralid moth, *Carposina niponensis* Walsingham (Shimizu *et al.* 1991), and the noctuids *Mamestra brassicae* Linnaeus and *Spodoptera littoralis* (Boisduvalis) (Tanada and Kaya 1993).

It has been estimated that the genome of *P. fumosoroseus* consists of six chromosomes, for a total size of 30.1 Mbp (Shimizu *et al.* 1991).

Pathogenicity of *Paecilomyces* spp.

Provided environmental conditions are suitable, fungal spores (*i.e.*, conidia) that come into contact with the insect host integument, germinate and via mechanical force and enzymatic activity, penetrate the host cuticle (McCoy 1974; Starnes *et al.* 1993). The serological properties of proteases involved in penetration of the cuticle have been examined for *P. fumosoroseus* (Shimizu *et al.* 1993). An appressorium is produced, and yeastlike hyphae (blastospores) proliferate by budding, using hemolymph as a food source. Death of the insect occurs mostly by mechanical displacement (Martens 1993), although secondary metabolites, such as beauvericin and leucinostatins, produced by the fungus may be involved (Onions 1979; Hajek and St. Leger 1994).

Host defense by an insect is effectively restricted to the integument; epicuticular lipids (*e.g.*, caprylic and capric acids) may be involved in the inhibition

of invasion by *P. fumosoroseus* in the silkworm moth, *Bombyx mori* (Linnaeus), and the fall webworm, *Hyphantria cunea* (Drury) (Saito and Aoki 1983).

The first symptoms of infection, apparent 24 to 48 hours after conidial contact with the insect cuticle, may include: visible color change of the host insect, mycelial growth between the head and prothorax, hyphae present in insect hemocoel, and hyphal growth eventually covering the entire surface of the host (Osborne and Landa 1992).

Potential Impact as a Biological Control Agent on *Bemisia* spp.

Paecilomyces spp. are registered and currently being used as microbial control agents against whiteflies, caterpillars, beetles, planthoppers and nematodes in the Philippines (Roberts and Hajek 1992). Results of field trials using *Paecilomyces* spp. against *B. tabaci* include: Inability to produce epizootics due to high mortality from UV light, wind, low humidity, and lack of sporulation in the field. The fungus was found to last up to 3 days (d) when sprayed as an inundative innoculation at 4 d intervals (Jaronski 1993, pers. comm.). Infectivity and commercial use will be enhanced if formulations can be produced that provide moisture retention and allow fungal growth at suboptimal relative humidity levels (Starnes *et al.* 1993).

McCoy *et al.* (1974) reported that the following four factors must be considered with respect to the efficacy of *Paecilomyces* spp.: dispersal, virulence, inoculum size, and viability. Dispersal of conidia is usually accomplished by wind,

although infected host movement and rain may also be involved. It has been noted that different isolates of *Paecilomyces* spp. may differ in virulence; *i.e.*, their pathogenicity to insect hosts (Fransen 1990). Such differences may be explained in part by heterokaryosis, anastomosis, and saprobic growth between host insect encounters (Roberts and Yendol 1971). Accurate determination of minimum inoculum size (measured as LD_{50}) necessary to induce disease in the field, which ought to be considered in any biological control program, is problematical (Roberts and Yendol 1971). And finally, viability may be influenced by the following factors: temperature, humidity, production of conidia and mycelia fragments on or in the host (Roberts and Yendol 1971; McCoy 1974).

Paecilomyces farinosus has been reported on *Bemisia tabaci* in India, and kills its host within 3 to 4 days (Asari *et al.* 1977). This species is the most common etiologic agent in sawflies, cerambycids, and pine shoot moth larvae (McCoy 1974). Virulence has been increased for *P. farinosus* by successive passes through insect hosts (Aizawa 1971). *P. farinosus* shows some saprophytic properties, which may enable this fungus to survive on forest duff in the absence of insect hosts (Harney and Widden 1991). Onillon (1990) reported a 90% mortality rate of *Bemisia tabaci* in the laboratory using *P. farinosus*, and noted this fungus' effectiveness against *B. tabaci* on cassava in India.

P. fumosoroseus infects all stages of *B. tabaci*, and some isolates (*viz.* the University of Florida patented isolate, PFR610) appear to be tolerant of pesticides (Becker *et al.* 1992). This latter quality is atypical among entomopathogenic fungi,

which are generally adversely affected by pesticides (Clark *et al.* 1982). This fungal species has excellent potential for incorporation into an IPM program due to its possible tolerance of pesticides, and its compatibility with other natural enemies, such as *Eretmocerus* spp., *Delphastus pusillus* (Osborne and Landa 1992), *Geocoris* sp. and *Chrysoperla* sp. (Jaronski and Hoelmer 1995).

Taxonomy

Bemisia spp.

Class Insecta (=Class Hexapoda)

Order Homoptera

Family Aleyrodidae

Bemisia tabaci Gennadius

Bemisia argentifolii Bellows and Perring.

Paecilomyces spp.

Division Eumycota

Subdivision Deuteromycotina (=Class Imperfecti)

Form-class Deuteromycetes (=Class Hyphomycetes)

Subclass Hyphomycetidae

Order Moniliales

Family Moniliaceae (Griffin 1994)

Paecilomyces farinosus (Holm ex Gray) Brown and Smith

Paecilomyces fumosoroseus (Wize) Brown and Smith.

Isozyme Analysis

Since the presence of isozymes was first reported by Markert and Möller (1959), their use in starch gel electrophoresis has proven to be an effective and powerful tool for studying the genetics of insects, such as *Bemisia tabaci* (Gill 1992; Bellows, Jr. *et al.* 1994), mammals (*e.g.*, Hartl *et al.* 1990), fish (*e.g.*, May *et al.* 1979b), bivalves (*e.g.*, Ayala *et al.* 1973), protozoa (*e.g.*, Guerrini *et al.* 1992), and fungi (*e.g.*, Moorhouse and de Bertoldi 1975; May *et al.* 1979a; Hellman and Christ 1991; Newton 1991; Elias and Schneider 1992; Leuchtmann *et al.* 1992; Simcox *et al.* 1993), including the entomopathogenic fungi *Metarhizium anisopliae* (Metsch.) Sorokin (de Conti *et al.* 1980; St. Leger *et al.* 1992b) and *Beauveria* spp. (Hajek and St. Leger 1994).

The technique of starch gel electrophoresis works on the principle of separating different forms of enzymes (proteins) based on their relative differences in net charge. These differences are due to the abundance and distribution of charged amino acids exposed to the gel matrix when subjected to a unidirectional electric current. This technique provides a conservative estimate of actual genetic variability

because only approximately one third of all different possible forms of an enzyme possess net charges sufficiently different as to be detected (Bonde *et al.* 1993). This is because there are only five amino acids (arginine, aspartic acid, glutamic acid, histidine, and lysine) which have ionizable side chains (Suzuki *et al.* 1981).

Based upon polymorphic loci, a number of distinct enzymes may be examined cumulatively to form a unique "fingerprint" of the operational taxonomic unit (OTU), which in the case of fungi is usually at the species level. A main advantage to using this technique over standard morphological characters, such as color, is a direct link between phenotype and genotype; the electromorph, or electrophoretic phenotype (zymogram), is an expression of enzyme structure (detected by differential electrophoretic mobility), directly determined by amino acid sequences, which are in turn directly coded for by DNA (Utter *et al.* 1987).

Polyacrylamide gel electrophoresis (P.A.G.E.) has also been used extensively in recent years to study fungal isozymes (*e.g.*, Anne and Peberdy 1981; Cruickshank 1983; Hodges, Jr. *et al.* 1986; Riba *et al.* 1986; Pitt *et al.* 1990; Damaj *et al.* 1993; Larsson 1994), including the biological control agent *Beauveria bassiana* (Bridge *et al.* 1990), but that technique has the disadvantages of higher cost and fewer enzyme systems that may be examined in a single electrophoretic run. However, resolution of banding patterns is often improved by use of this matrix due to separation of enzymes based on their size as well as net charge (Bunnell 1994, unpubl. data). Other electrophoretic techniques, such as disc electrophoresis of salt soluble proteins, acid-phenol electrophoresis of whole cells, split-gel systems, and isoelectric focusing used

in fungal taxonomic studies are discussed by Chesson *et al.* (1978) and Micales *et al.* (1992).

Methods other than isozyme analyses useful in fungal systematics (*e.g.*, DNA studies using RFLPs, PCR, and G-C content) are discussed in Klich and Mullaney (1992), in a review by Kohn (1992), and in Bidochka (1994). These methods are more suitable for detecting differences at the intraspecific level, whereas the differences in genomes of a significant enough nature as to be detected by starch gel electrophoresis are usually found between species (Bonde *et al.* 1993).

The following fungal growth culture condition variables may potentially affect observed electromorphs, and must therefore be held constant for a given analysis: media, *e.g.*, sources of carbon and nitrogen (glucose, maltose, etc.), physiological state (mycelium vs. blastospore), and age (early-mid logarithmic phase vs. stationary phase) (Jaronski 1994, pers. comm.).

Other experimental variables with the potential for influencing electromorphs include pH of the gel and electrode buffers, voltage and temperature during electrophoresis, and age of samples. Bonde *et al.* (1993) reported no appreciable loss of enzymatic activity for samples stored at -80°C for at least one year.

Types of Isozymes

There are biological requirements for what may appear to be a redundant system of enzymes given the fixed energy budget of any organism. These include

certain metabolic cellular conditions in which a single reaction needs multiple forms of an enzyme for catalysis, and changing requirements over time or space (Markert 1975).

Different types of isozymes fall into the following categories: a. conformational isozymes, or conformers--different tertiary structures (folding) resulting in a different proportion of charged (amino or carboxyl) groups exposed; b. genetically determined (segregating) isozymes--due to allelic variation; c. nonsegregating isozymes--also different genetically, but bands are common to all members of the population; d. homopolymers--protein consisting of more than one identical subunit; e. heteropolymers--protein consisting of more than one type of subunit; and f. isokinetic isozymes--proteins sharing approximately the same quantitative activity (Brewer and Sing 1970).

In practice, however, it is convenient to simplify the classification of different detectable isozymes into three main groups: multiple alleles at a single locus determining different versions of the polypeptide chain (allozymes), multiple gene loci coding for different polypeptide chains of a single enzyme (isozymes), and those due to post-translational changes (Harris and Hopkinson 1976).

Specificity of enzymatic reactions

The high specificity of biochemical reactions taking place *in vitro* which results in the visualization of a product to be measured as a band on a gel is a result

of one of the following different staining techniques: a. simultaneous capture method; b. postincubation capture reaction; c. autochromic method; d. overlay ("sandwich type"); and e. copolymerization of substrate in gel (Heeb and Gabriel 1984).

3. MATERIALS AND METHODS

Fungal Growth Culture Conditions

Isolate codes, the original host, and the geographic origins for the 23 fungal isolates analyzed are presented in Table 4 (see Appendix). Samples were obtained from Mycotech Corp. (Butte, Montana) while the fungal isolates were in the haploid mycelial stage of assimilative growth. Mycelia were grown in 100.0 ml of Sabouraud-maltose-yeast (SMY) broth in glass flasks on a shaker for 10 days. They were then separated from the broth using grade 202 Rive Angel filter paper and vacuum suction. The mycelia were rinsed several times with distilled water, and the mycelial mat scraped off of the filter paper into a glass vial and frozen immediately at -25°C.

Sample Preparation

A 2X (volume : mass) enzyme extract buffer (0.5 M TRIS-HCl, pH 6.8) was added to the mycelia (*e.g.*, 500 μl buffer : 250 mg mycelium), and samples were crushed mechanically using a Virtis 23 tissue grinder. Capillary action then was used to draw the extracted enzymes into wicks cut from Whatman #4 filter paper. Extracted samples then were frozen and stored at -80°C.

Starch Gel Electrophoresis

12.0% gels were made by mixing 60.0 g hydrolyzed potato starch (Sigma Chemical Co. #S-4501) with 500.0 ml gel buffer (Table 5, Appendix) in a 1000.0 ml Erlenmyer flask. The flask was then constantly swirled over a bunsen burner flame. The solution became less opaque and noticeably thicker as the bubbles formed. Heating was continued until the solution became slightly thinner than at its thickest point. The flask was then removed from the flame, and the solution was immediately de-gassed (aspirated) with vacuum pressure until the bubbles formed were of more or less uniform size. The gel solution was then quickly poured into preformed gel molds, using disposable pipets to remove any remaining bubbles. The gel solution was allowed to cool at room temperature and covered with plastic wrap, while being careful to prevent any air bubbles between gel and plastic. The gel solution was then refrigerated at 4° C for at least one hour before loading the samples.

Wicks were spaced evenly along the origin slice in the gel (4.0 cm from the cathodal edge of gel). Starch gel electrophoresis was carried out at 4°C, 75-100 milliamperes, 45-60 V, for 16 hours. One marker wick using blue food coloring was used to monitor the progress of enzyme migration. Dye was allowed to travel 10.0 cm from the origin to the anodal edge of the gel. Gels were then sliced with nylon fishing leader (Berkley Trilene XL 2 lb. test, 0.01 cm dia.) into six 1.6 mm layers, so that a single electrophoretic run allowed six enzymes to be examined.

Enzyme Staining

Slices were stained according to the protocols outlined in Tables 3 and 4, and allowed to develop for *ca.* 30 minutes in a 37°C incubator.

Photodocumentation was obtained with a Nikon FM-2 mounted on a camera stand under 120 V halogen lights at 1/125 of a second shutter speed, f16 aperture opening with a red filter using 125 ASA Ilford black and white 35 mm film, or on an ultraviolet light table with a camera hood using Polaroid 667 film.

Scoring Bands

Measurements were made from the origin to a given band using electronic calipers to the hundredth of a mm. Bands of the most consistently staining isolate were designated a mobility of 1.0; *i.e.*, relative migration distance (R*f*) = 1.0. Other bands were assigned R*f* values based on their homomeric protein products' (alleles') positions relative to the standard, as described by May *et al.* (1979a).

Statistical Analysis

Genetic diversity provides a measure of the variability at a given locus for each fungal isolate. This statistic is analogous to average (intralocus) heterozygosity in diploid systems (Nei 1987). The statistical package GeneStat-PC 3.3 (Lewis 1992)

calculates these results, as well as provides variance estimates of the gene diversity statistics.

Nei's genetic distance, $D = - \ln I$ (Nei 1972), where I = standard genetic identity, for all pairwise combinations was calculated, based on allele frequency data, using the software package GeneStat-PC 3.3 (Lewis 1992). This distance measures the extent of gene differences between isolates (putative allele frequencies).

The quantity I, standard genetic identity, represents a ratio of the proportions of loci that are alike within and between isolates (Weir 1990).

The nearest neighbor method (neighbor-joining) cluster analysis of similarity coefficient matching was performed using the multivariate statistical package NTSYS-PC (Rohlf 1993). This procedure involved distance coefficients originally derived from binomial band presence/absence data. Also employed were the ordination procedures principle components analysis (PCA) and nonmetric multi-dimensional scaling (NMDS) (Rohlf 1993). The latter employed simple matching (SM) coefficients derived from a symmetric similarity matrix.

4. RESULTS

Screening Run

The thirty-four enzymes listed in Table 8 (Appendix) were used in the screening run with the nine isolates PF601, PF602, PF603, PF604, PFR600A, PFR601, PFR602, PFR603, and PFR604. Of these, twelve produced consistent bands of relatively uniform intensity showing differences between the two species, nine produced consistent bands which provided no ability to discriminate between species, and twelve failed to provide clear, consistent banding patterns. As in Rakotonirainy *et al.* (1994), banding data was recorded irrespective of intensity.

Molecular Markers for Distinguishing Between Two Species

The following twelve enzyme-buffer systems (listed in Table 9, Appendix) were useful as molecular markers (diagnostic tools) for differentiating between the two species: AC, ADH, AGP, CAR, DIA, G6PDH, GP, GPI, GR, HBDH, MPI, and PGM (see Tables 6 and 8, Appendix, for names of enzymes corresponding to the abbreviations just given). Figure 1 shows a diagrammatic, composite representation of the electromorphs corresponding to Table 9 (Appendix). Figure 2 shows an example of an actual gel (enzyme-buffer system GPI), and the readily apparent differences between the two *Paecilomyces* species.

Figure 1. Composite zymogram based on electrophoretic phenotypes corresponding to those listed in Table 9 (Appendix). Names of enzymes abbreviated along the x-axes are listed in Tables 6 and 8 (Appendix).

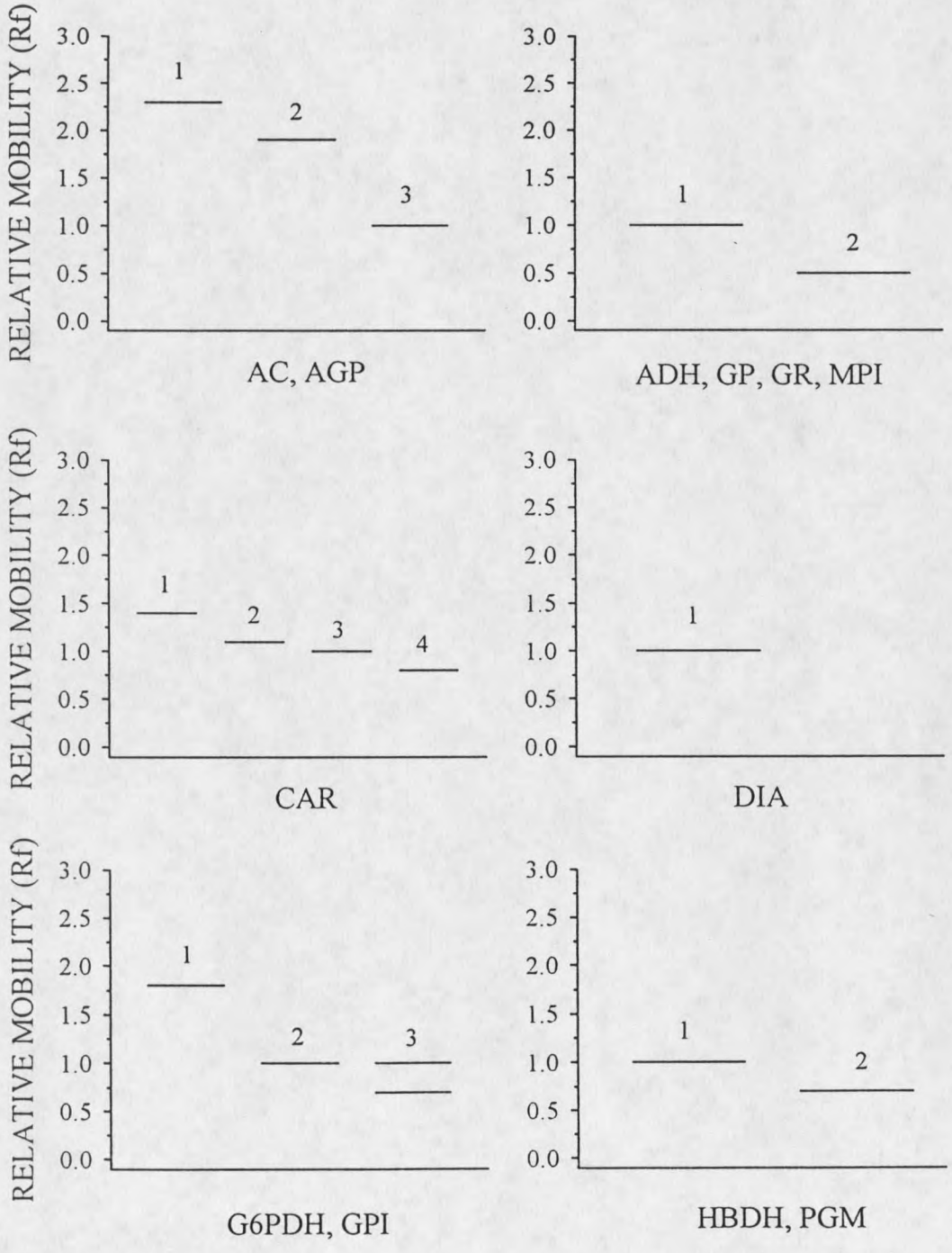
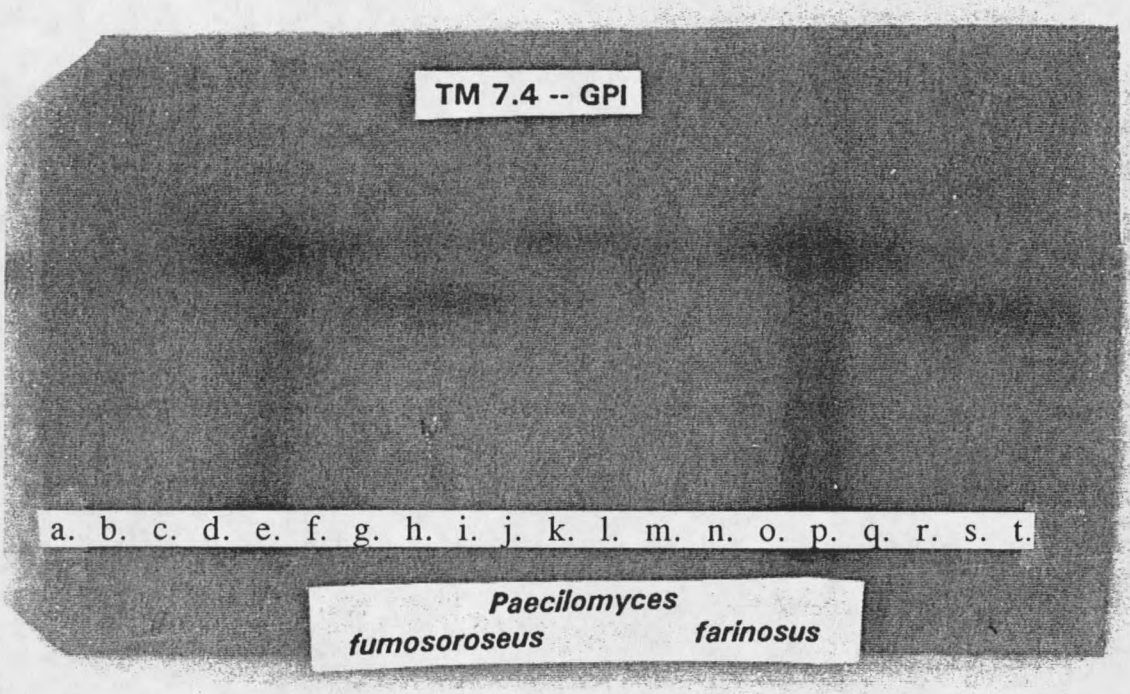


Figure 2. Photograph of actual gel, enzyme-buffer system TM 7.4 (Table 5, Appendix), isozyme GPI (E.C. 5.3.1.9) (Table 6, Appendix). Black and white 35 mm Ilford film under halogen lights (1/15 sec., f8), 22 July 1994. Lanes are identified by isolate codes (see Table 4, Appendix).



a, k. PFR600A
b, l. PFR600A
c, m. PFR603
d, n. PFR603
e, o. PFR610
f, p. PFR612
g, q. PFR612
h, r. PF601
i, s. PF601
j, t. PF609

The only distinguishable isolates within species were *P. farinosus* PF601, and PF606 with the enzyme-buffer systems AGA and HEXA; *P. fumosoroseus* isolates PFR605 and PFR609 with the enzyme-buffer systems AC, ADH, AGP, AK, FDP, GAPDH, GK, and HBDH; and PFR623 using the enzyme-buffer system CAR.

Estimated Genetic Diversity Among All Isolates

Twenty-three enzyme-buffer systems produced thirty-nine loci (consistent banding patterns, or electromorphs) for all twenty-three isolates. Only band loci (both polymorphic and nonpolymorphic) common to both species of *Paecilomyces* were included in this portion of the analysis. The R*f* values for the discrete loci are listed in Table 1. Two "alleles," A and B, for each locus represent the presence or absence, respectively, of a band at that relative migration distance (R*f*). The mean number of individual samples tested per locus was 5.76.

Mean values of sample genetic diversity for each isolate over all loci, with standard errors, are shown in Table 2, and the raw statistics are presented in Table 10 (Appendix). These tables represent intralocus variance. Nei (1987) points out that although the equivalent interpretation of this analysis as average heterozygosity does not apply to the haploid fungi studied herein, it is perfectly valid to test the probability of differences in randomly chosen genes from a population. It is noteworthy that while isolate PF601 showed the greatest genetic distance (see Nei's Genetic Distances below), this isolate did not demonstrate the greatest genetic

Table 1. Identification of band positions for common loci, measured as relative migration distance (*Rf*). Refer to Tables 6 and 8 for more information regarding locus (enzyme) abbreviations. Numerals following abbreviations designate discrete bands; the higher the value, the more anodal the band position.

Locus	*Rf*	Locus	*Rf*
AAT	1.0	IDH	0.5
AC	1.0	LDH-1	1.0
ADH-1	1.0	LDH-2	0.7
ADH-2	0.5	LDH-3	0.6
AK	1.0	LDH-4	0.5
DIA	1.0	MADH	1.6
FDP	1.0	MDH-1	1.0
G6PDH-1	1.8	MDH-2	0.8
G6PDH-2	1.0	ME-1	1.0
G6PDH-3	0.7	ME-2	0.7
GAPDH	1.0	ME-3	0.6
GK-1	1.0	MPI-1	1.0
GK-2	0.1	MPI-2	0.7
GP	1.0	PGM	1.0
GPI-1	1.1	SDH	1.0
GPI-2	1.0	SOD	1.0
GPI-3	0.9	XDH-1	1.0
GR	1.0	XDH-2	0.1
HBDH-1	1.0		
HBDH-2	0.7		
HBDH-3	0.5		

Table 2. Sample genetic diversity for each isolate averaged over all thirty-nine loci, with standard errors. This table provides estimates of gene diversity which are used to evaluate the significance between isolates. See Table 4 (Appendix) for more information concerning isolates. Calculations performed by GeneStat-PC (Lewis 1992).

Isolate	code	mean diversity	S.E.	Isolate	code	mean diversity	S.E.
PF601	F1	0.1936	0.0305	PFR604	R4	0.1929	0.0341
PF602	F2	0.2114	0.0336	PFR605	R5	0.0263	0.0263
PF603	F3	0.2278	0.0330	PFR606	R6	0.0000	0.0000
PF604	F4	0.1427	0.0301	PFR607	R7	0.0500	0.0344
PF606	F6	0.0222	0.0222	PFR609	R9	0.0000	0.0000
PF607	F7	0.0694	0.0380	PFR610	R10	0.1129	0.0381
PF608	F8	0.0722	0.0395	PFR611	R11	0.0250	0.0250
PF609	F9	0.0315	0.0219	PFR612	R12	0.0607	0.0292
PFR600A	R0	0.1946	0.0339	PFR613	R13	0.1416	0.0498
PFR601	R1	0.2412	0.0321	PFR621	R21	0.0250	0.0250
PFR602	R2	0.2528	0.0320	PFR623	R23	0.0000	0.0000
PFR603	R3	0.1888	0.0335				

diversity, nor corresponding standard error. This finding casts doubt on experimental error as being responsible for the observed relatively high genetic variability.

The greatest value for mean genetic diversity measured corresponded to isolate PFR602 at 0.2528, while the lowest value, 0.0000 occurred thrice: isolates PFR606, PFR609, and PFR623. The highest standard error observed corresponded to isolate PFR613 at 0.0498, while the lowest value, 0.0000, was again found in the three isolates which showed no diversity (as measured to four decimal places) mentioned above (PFR606, PFR609, and PFR623).

Similarity Indices

Results of principle components analysis (PCA) are displayed two-dimensionally in Figure 3, and three-dimensionally in Figures 4a and 4b. The first three principle axes (eigenvalues) account for 64.6% of the variation in the data. Figure 5 displays results of nonmetric multidimensional scaling (NMDS) in good agreement with those derived from PCA (both analyses performed with NTSYS; Rohlf 1993). Both methods produced two loosely defined clusters: The tighter of the two consisted mostly of *P. fumosoroseus* isolates, and the more spread out group consisted of *P. farinosus* isolates PF601, PF602, PF603, and PF604, and *P. fumosoroseus* isolates PFR600A, PFR601, PFR602, PFR603, and PFR604. PF601 was positioned in three-dimensional space further from any other isolate (Figures 4a and 4b).

Figure 3. 2-Dimensional plot of principle components analysis (PCA) results. x- and y-axes(eigenvalues) account for 54.44% (42.14% and 12.30%, respectively) of the variation in the data. See Table 2 for explanation of isolate codes. Statistical package used: NTSYS-PC (Rohlf 1993).

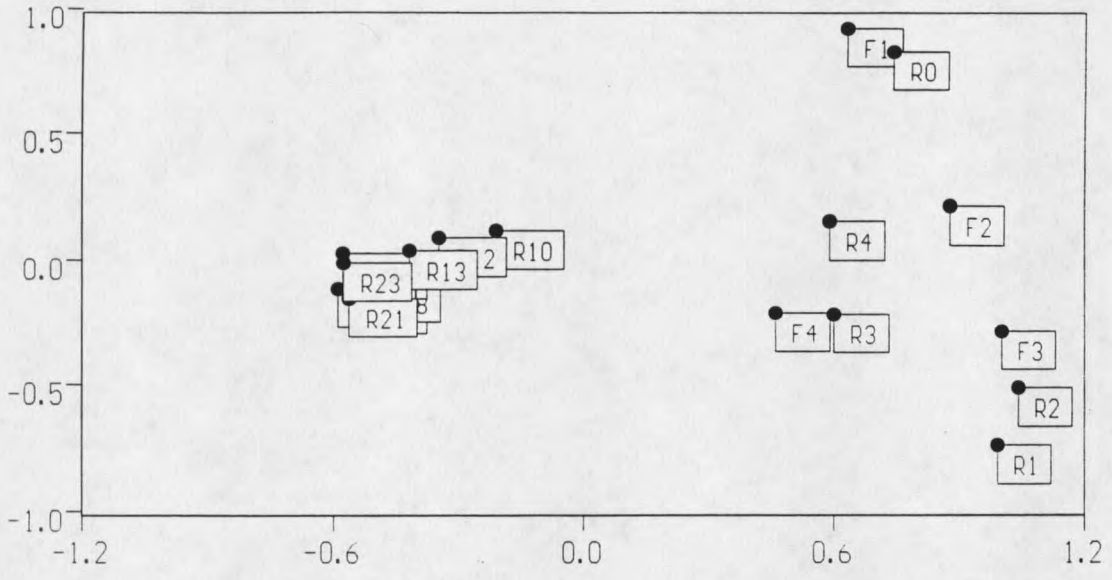


Figure 4a. 3-Dimensional graph of principle components analysis (PCA). Balls at the end of points are identified in Figure 4b. Calculations performed by NTSYS-PC (Rohlf 1993).

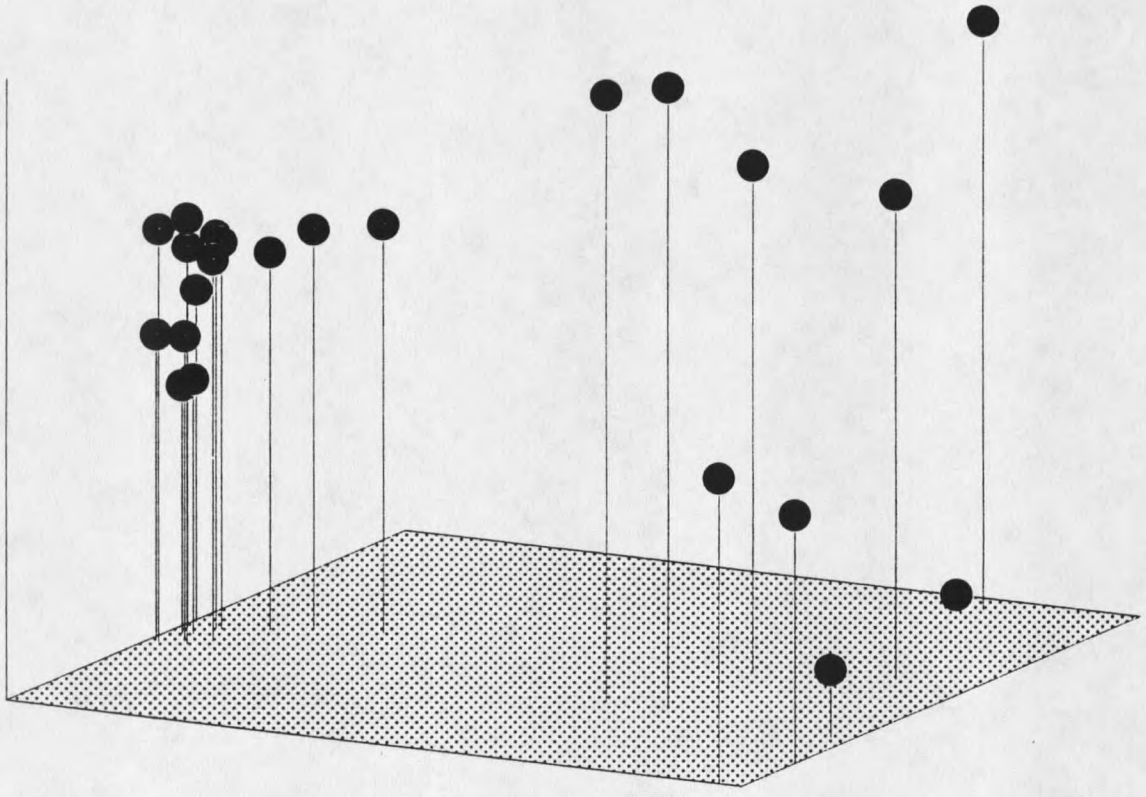


Figure 4b. 3-Dimensional graph of PCA. "F" and "P" prefixes refer to *P. farinosus* and *P. fumosoroseus*, respectively, as detailed in Tables 2 and 4 (Appendix). Calculations performed by NTSYS-PC (Rohlf 1993).

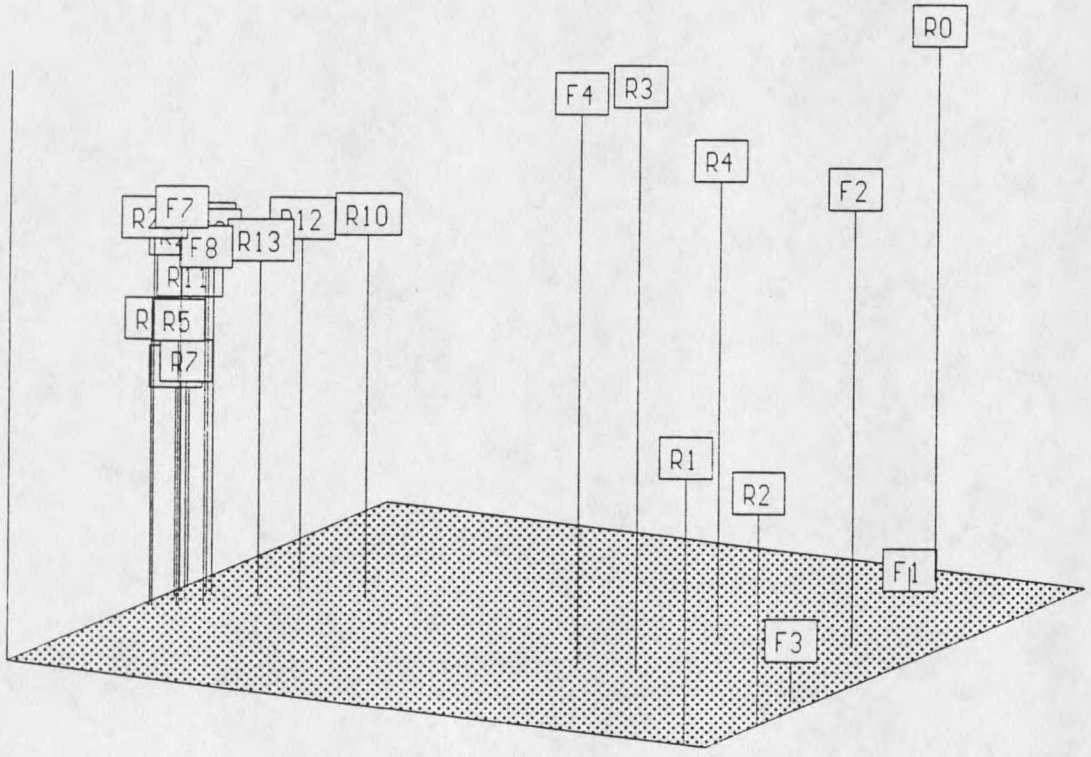


Figure 5. Results of nonmetric multidimensional scaling as calculated by NTSYS-PC (Rohlf 1993). "F" and "R" prefixes refer to *P. farinosus* and *P. fumosoroseus*, respectively, as detailed in Tables 2 and 4 (Appendix).

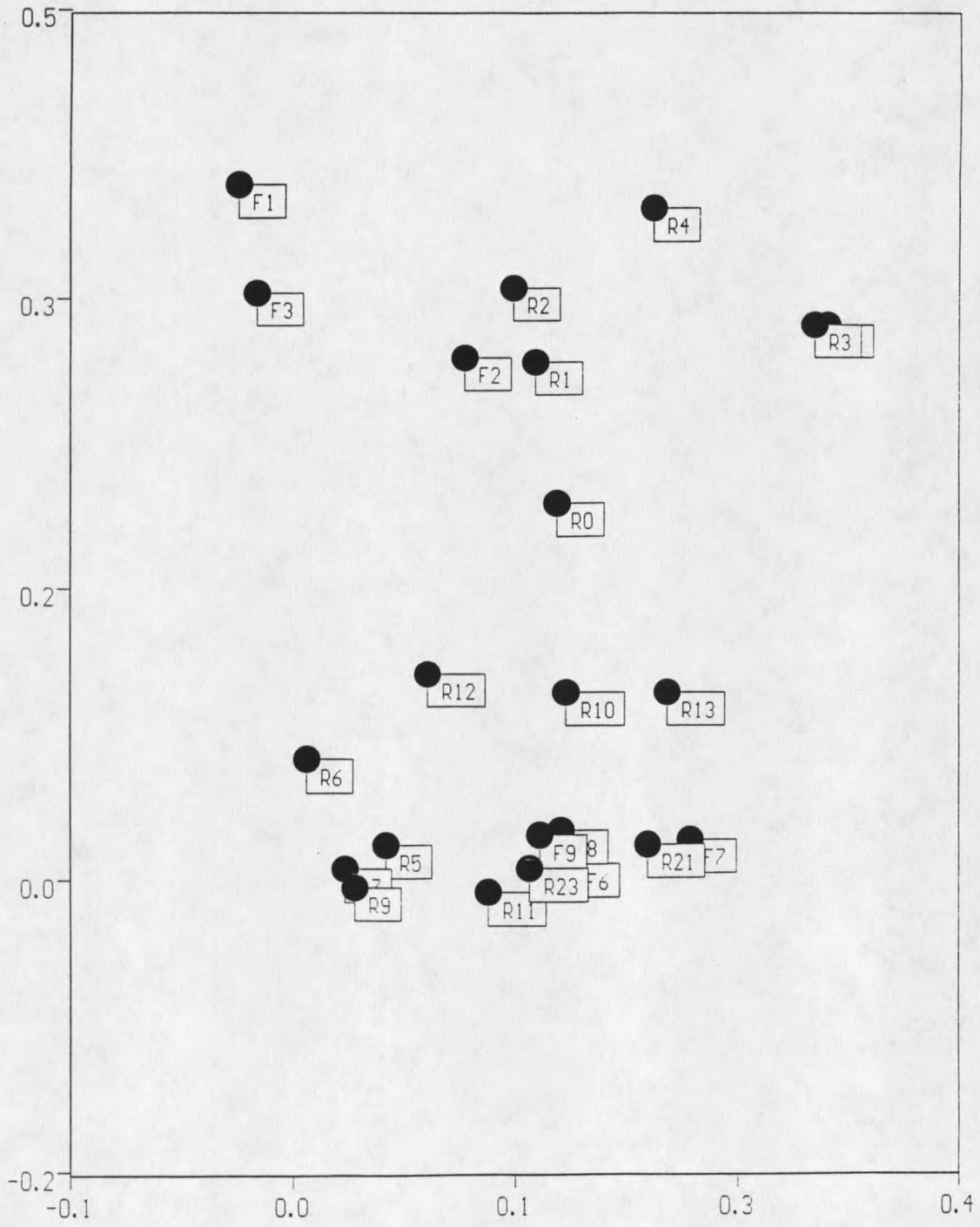
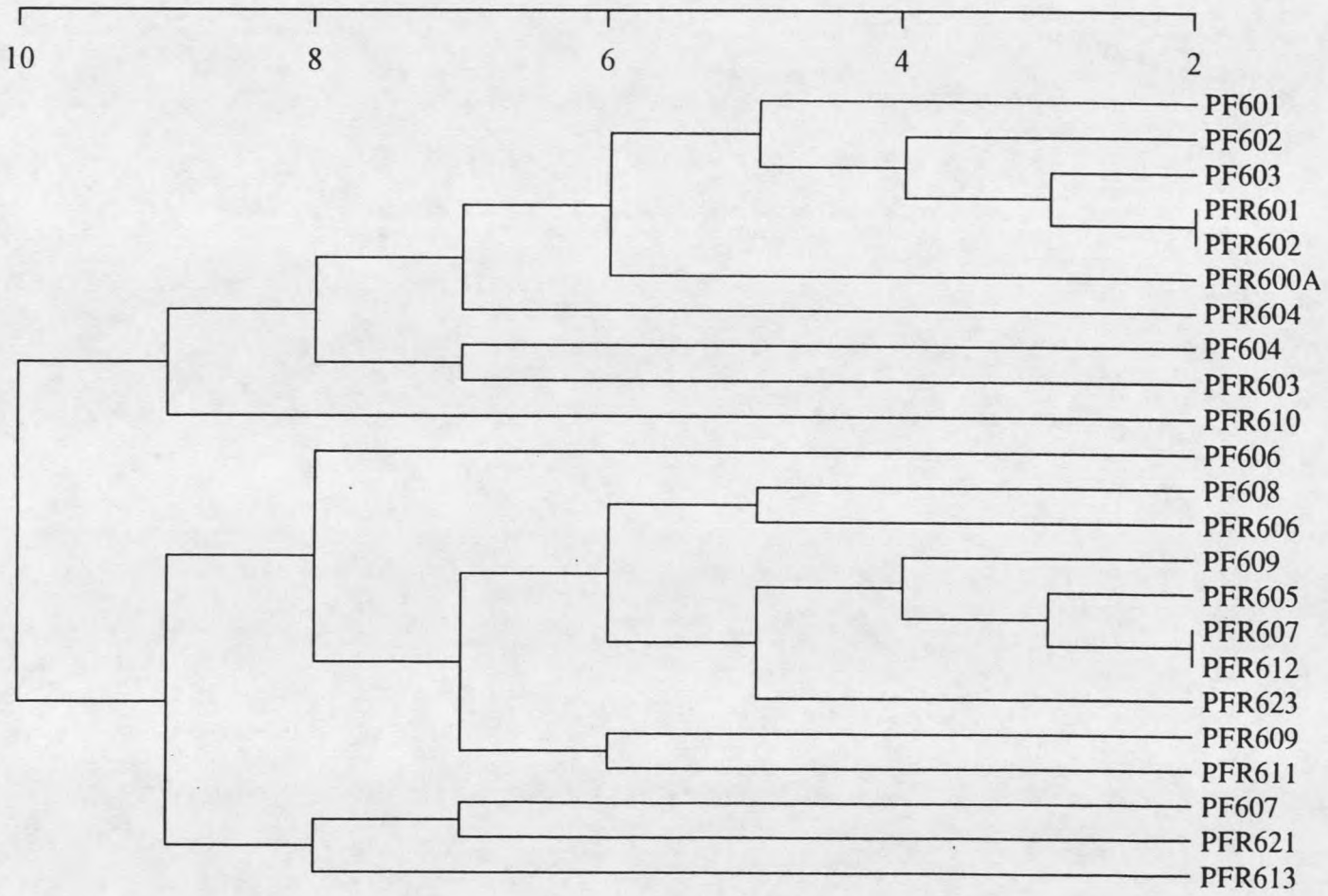


Figure 6. Genetic distance dendrogram, based on Nei's (1972) genetic distances using nearest neighbor joining method. Refer to Table 4 (Appendix) for explanation of isolate codes, which appear at the end of branches. Calculations performed by NTSYS-PC (Rohlf 1993).



The two French *P. fumosoroseus* isolates, PFR601 and PFR602 display minimal genetic distance from each other as shown in the dendrogram (Figure 6). In contrast, two other isolates collected from the same field in Texas on the same day (PFR605 and PFR606) do not appear, on the basis of similarity of distance coefficients, to be as closely related as the isolates PFR607 and PFR612, the former from California and the latter Texas.

As mentioned earlier (*Paecilomyces* spp., Identification, p. 8), *P. fumosoroseus* isolate PFR600A (isolated from *Bemisia* spp. from Texas) has been identified as a *P. farinosus* isolate. This isolate falls out in the same cluster as one containing three *P. farinosus* isolates and two other *P. fumosoroseus* isolates in the dendrogram (Figure 6). PFR600A also appears to bridge the two main clusters to some extent in Figure 5.

Nei's Genetic Distances

Mean genetic distances and mean genetic identities for each isolate over all others are summarized in Table 3, while the raw values of those statistics are presented in Table 11 (Appendix); the latter of which is a symmetric table showing genetic distances and identities for each pairwise isolate comparison. The statistical package GeneStat-PC was used to generate these values (Lewis 1992).

PF601, the *P. farinosus* isolate from the Czech Republic (from *Leptinotarsa decemlineata*) showed the greatest genetic distance as displayed in Table

Table 3. Mean genetic distances and genetic identities for each isolate of *P. farinosus* (PF prefix) or *P. fumosoroseus* (PFR prefix) over all others.

Isolate	D^1	I^2	Isolate	D^1	I^2
PF601	0.2069	0.8149	PFR604	0.1455	0.8664
PF602	0.1355	0.8748	PFR605	0.1090	0.8982
PF603	0.1474	0.8638	PFR606	0.1995	0.8215
PF604	0.0790	0.9249	PFR607	0.1495	0.8631
PF606	0.1034	0.9041	PFR609	0.1427	0.8691
PF607	0.0968	0.9085	PFR610	0.1420	0.8694
PF608	0.1179	0.8911	PFR611	0.1088	0.8990
PF609	0.1015	0.9044	PFR612	0.1242	0.8840
PFR600A	0.1298	0.8801	PFR613	0.1266	0.8834
PFR601	0.1074	0.8993	PFR621	0.1289	0.8814
PFR602	0.0911	0.9138	PFR623	0.1025	0.9046
PFR603	0.0617	0.9408			

1. D = Nei's genetic distance, D = - ln$I$ (Nei 1972).

2. I = standard genetic identity, which represents a ratio of the proportions of loci that are alike within and between isolates (Nei 1972; Weir 1990).

10 with a mean value of 0.2069. In contrast, the least genetic distance was displayed by isolate PFR603 at a mean value of 0.0617. Similarly, PF601 and PFR603 demonstrated the least and the greatest values of genetic identity, respectively, at 0.8149 and 0.9408.

The results of cluster analysis using presence or absence of a band at each locus as allele frequency data were used to construct the dendrogram in Figure 6. This method, based on Nei's genetic distances used as coefficients for nearest neighbor joining, shows a high level of correspondence with the PCA and NMDS results, both of which analyses do not require previous assumptions about the data, such as correct identification of the isolates in question.

It is apparent that the isolates of the two species did not clearly group into two discrete clusters. Two main groups emerge: One consisting of four *P. farinosus* isolates and four *P. fumosoroseus* isolates, and the other contains four *P. farinosus* isolates and ten *P. fumosoroseus* isolates, with isolate PFR600A bridging, to some extent, the two loose groups. This bridging is most apparent in the first (x-) axis of the PCA (eigenvalue accounting for 42.14% of the variation in the data set), and, graphically, by NMDS (see Figure 5). The two isolates furthest from each other at the ends of the dendrogram branches were PF601 and PFR613 (see Figure 6).

5. DISCUSSION

Isozyme analysis useful to "fingerprint" species

While isozyme analysis has demonstrated its usefulness in differentiating the two species of *Paecilomyces* included in this study as a diagnostic tool, this technique has limitations for elucidating intraspecific genetic variability due to its conservative nature. Because of ambiguities in genetic interpretation of fungal electrophoretic polymorphisms (*e.g.*, Leuchtmann *et al.* 1992; Damaj *et al.* 1993), simple presence-absence band counting techniques have been endorsed (Micales *et al.* 1992). Assignment of specific alleles and loci to bands must remain putative at best because traditional crossing experiments which might clarify the underlying causes of multiple bands for a given enzyme (see Types of Isozymes, p. 17) are impossible in the haploid, asexual fungi studied (Elias and Schneider 1992).

Low genetic variability detected

Based on observed electromorphs, the relative genetic variability within each of the *Paecilomyces* spp. examined using isozyme analysis as a means of measurement is minimal. In nine of the enzyme-buffer systems with consistent banding patterns, there was no observable difference among any of the isolates' zymograms (AAT, AK, FDP, IDH, LDH, MDH, ME, SDH, and SOD).

Cluster analysis resolved the 23 isolates into loosely defined but distinct groups, providing evidence of nonrandom segregation of loci. The power of isozyme analysis appears to be minimal, however, for this purpose as evidenced by the mixing of isolates from both species within clusters. Nei (1987) cites unique mutation, random genetic drift, epistatic selection, and migration as possible sources of the observed variability.

The inclusion of nonpolymorphic loci as well as polymorphic ones provided more valuable information than previous studies using only polymorphic loci (*e.g.*, St. Leger *et al.* 1992a; St. Leger *et al.* 1992b) due to the predictive power of this method if no prior taxonomic information is available (Zeng 1995, pers. comm.). While those authors qualified their results as *relative* genetic similarity, this is already implied in the random selection of genes (loci) analyzed (Nei 1987).

In comparison to studies on the entomopathogenic fungi *Beauveria* spp. and *Metarhizium* spp. (*e.g.*, St. Leger *et al.* 1992a; St. Leger *et al.* 1992b), *P. farinosus* and *P. fumosoroseus* demonstrate an overall lack of correspondence between genetic similarity as measured by isozyme analysis and similarity of geographic origin. However, this is probably influenced largely by the lack of inclusion of valuable no-difference data as discussed above in those studies.

Paecilomyces farinosus 601

Isolate PF601, isolated from *L. decemlineata* in the Czech Republic, demonstrated the greatest mean genetic distance from all other isolates at D = 0.2069. In contrast, estimated gene diversity for this isolate does not display the greatest variance (mean genetic diversity for all loci = 0.1936, S.E. = 0.0305). For example, PFR602 demonstrated the highest estimated gene diversity (mean = 0.2528, S.E. = 0.0320) (see Table 2). The lowest value observed was 0.0000 for PFR609 (S.E. = 0.0000). This information, combined with the zymogram data, suggest one of several possibilities for this isolate: an unusually high degree of genetic differentiation from other conspecifics, contamination of starting culture material, misidentification, or the existence of a new species (Munsterman 1994, pers. comm.).

Heterokaryosis

Nuclear staining and genetic analyses have confirmed heterokaryosis in *Paecilomyces farinosus* (Liu and Wu 1992). The selective advantage of heterokaryotic organisms over homokaryotic strains is due to the former having more balanced genetic systems (Elander and Lowe 1992). While the level of enzyme polymorphism is generally expected to be lower for haploid fungi than for diploid plants and animals, it is not universally so (Garber 1973). Heterokaryosis offers partial explanation for greater degrees of polymorphism than might be expected; *e.g.*,

potentially lethal mutations may be preserved in the vegetative mycelial state (Garber 1973). Moreover, in a hererokaryotic system, genetically distinct nuclei are known to fuse and recombine (Burnett 1968). While hyphal fusion plays a prominent potential role in bringing together dissimilar genotypes, isolating mechanisms or sterility barriers do exist which act to minimize such effects (Burnett 1968).

Conclusion

Isozyme analysis has been shown to be an effective means of distinguishing between the two species *P. farinosus* and *P. fumosoroseus*, using the representative isolates chosen for inclusion in this study. However, this technique does not appear to be effective for discrimination at the intraspecific level.

Cluster analysis indicated one of at least two possibilities: a. a revision of the genus is in order; or b. isozyme analysis is not an adequate method to generate data useful in representing a true evolutionary history of the isolates examined.

Recent work by Tigano-Milani *et al.* (1994) using AP-PCR data from fungal cultures that had been allowed to grow for 3 days suggested that some isolates of *Paecilomyces fumosoroseus* obtained from *Bemisia tabaci* are dissimilar enough to raise the possibility of revision of the species.

Based on the results of this study, more work needs to be done in order to further clarify probable phylogenetic relationships among isolates within these two *Paecilomyces* species. Recommendations include: examining more isolates from a

wider variety of geographic locations; recording host plant, relative humidity (RH), temperature, sunlight exposure, and other climatic and edaphic data corresponding to the insect hosts and geographic locations from which the fungi are isolated, allowing studies of potential tri-trophic interactions, and how they may influence fungal genetics and selection; inclusion of additional genetic and physiological data, such as DNA analyses (*e.g.*, directed PCR with functional gene sequences as targets, RAPD-PCR, or conserved ribosomal RNA sequences) which potentially offer greater resolution of differences at the intraspecific level (*e.g.*, Curran *et al.* 1994; Rakotonirainy *et al.* 1994; Zimand *et al.* 1994); and examining samples grown under varying culture conditions (*e.g.*, length of time/growth phase, media).

While these isozyme results do not provide a clear interpretation of the phylogenetic relationships within these two *Paecilomyces* spp., they do provide valuable information in terms of diagnostic profiles and in furthering our understanding of this genus, about which much less is known, biochemically and taxonomically, than other fungal biological control agents such as *Beauveria* spp. (*e.g.*, St. Leger *et al.* 1992a) and *Metarhizium* spp. (*e.g.*, St. Leger *et al.* 1992b).

These preliminary investigations regarding the molecular genetics of *Paecilomyces* spp., a pathogen with a wide host range, contribute to the development and refinement of microbial insecticides, and their use in large-scale agriculture. Collaboration among other laboratories, for example the U.S.D.A.-A.R.S. Plant Protection Unit in Ithaca, New York, which is also looking at the molecular genetics (RAPD-PCR, see glossary) of *P. fumosoroseus* isolates PFR602 and PFR604

(Cantone 1995, pers. comm.), will undoubtedly expedite the expansion of basic knowledge about these fungal species. This, in turn, ought to facilitate the incorporation of these mycoinsecticides into successful IPM programs. If future work involving fungal molecular biology, encapsulation technology, genetic manipulation (transformation systems and recombinant DNA technology), or other ways of increasing the efficacy of *Paecilomyces* spp. is carried out, these fungi may someday help increase the market share of microbial insecticides (biologicals) to at least 1% of the total insecticides used in pest control (Starnes *et al.* 1993).

6. SUMMARY

Reliable identification of fungal biological control agents is critical due to concerns such as differences in efficacy, protection and maintenance of patentable lines, and release and redistribution of approved isolates only. Because the identification of fungal isolates by morphological observation is often inconclusive, molecular marker techniques may provide an alternative and more reliable method for fungal identification. Currently, the literature is bereft of molecular markers for *Paecilomyces* spp., unlike the case with other fungal biological control agents such as *Beauveria* spp. and *Metarhizium* spp.

Twenty-three isolates were selected from the genus *Paecilomyces*, eight *P. farinosus* and fifteen *P. fumosoroseus*, for inclusion in the present study. All isolates were grown under uniform culture conditions, enzymes were extracted, and separation was achieved by starch gel electrophoresis using thirty-four enzyme-buffer system combinations.

A battery of twelve enzyme-buffer systems producing only polymorphic loci was effective and efficient at consistently distinguishing between the two species of fungus. Data analysis, applying multivariate statistical methods to *all* data (polymorphic and non-polymorphic loci), indicated relatively low genetic variability and an ambiguous separation into groups based on species differences.

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APPENDIX

Tables 4-11

Table 4. The 23 *Paecilomyces* spp. fungal isolates used in this study. Information given includes isolate code original insect host from which sample was collected, and geographic origin (when available). See also Table 2 for other isolate codes used in Figures and Tables.

Species	Mycotech #	ARSEF[1] #	ATCC[2] #	SARL[3] #	host	geographic origin
P. farinosus	601		26853		*Leptinotarsa decemlineata*	Czech Republic
(also referred to	602	3			*Malacosoma americanum* (Eastern tent caterpillar)	Mass., U.S.A.
in text as "PF,"	603	3564			*Taneo* spp. (thrips)	Vermont, U.S.A.
or "F" prefix).	604	3526 ("3562")			*Lymantria dispar*	Maryland, U.S.A.
	606	3522			*L. dispar*	W. Virginia, U.S.A.
	607	20			*L. dispar*	Pennsylvania, U.S.A.
	608	UVt 3731			Pear thrips	Vermont, U.S.A.
	609	UVt 3741			Pear thrips	Vermont, U.S.A.
P. fumosoroseus	600A	3572			*Bemisia tabaci*	Texas, U.S.A.
(also referred to	601		42188		*Mamestra brassicae*	France
in text as "PFR,"	602	1644			*Musca domestica*	France
or "R" prefix).	603	2744			*Tenebrio* spp.	Philippines
	604	3083			*Bemisia* spp.	Florida, U.S.A.
	605	3581			*Bemisia* spp.	Texas, U.S.A.
	606	3594			*Bemisia* spp.	Texas, U.S.A.
	607	3658			*Bemisia* spp.	El Centro, Calif., U.S.A.
	609	3663			*Bemisia* spp.	Calexico, Calif., U.S.A.
	610		20874		*Bemisia* spp.(?)	Florida, U.S.A.
	611			16	*Bemisia* spp.	--
	612			47	--	Weslaco, Texas, U.S.A.
	613			48	*Bemisia* spp.	Weslaco, Texas, U.S.A.
	621	3076		149	*Bemisia* spp.	Florida, U.S.A.
	623	1569		148	*Hyphantria cunea*	Italy

1. United States Department of Agriculture (U.S.D.A.)-Agricultural Research Service (A.R.S.) Entomopathogenic Fungi (E.F).
2. American Type Cuture Collection (ATCC).
3. U.S.D.A. Southern Area Research Lab (SARL), Weslaco, Texas.

Table 5. Gel/electrode electrophoresis buffer systems used in this study. Protocols have been adapted from those described in references following system name parenthetically.

name	reagent	quantity	source
TM 7.4 (Pasteur *et al.* 1988):			
• Solution I	Tris (0.1 M)	24.2g	Sigma T-1503
	Malic anhydride (0.08 M)	16.2g	Sigma M-1000
	H_2O, make up to	1000.0ml	
• Solution II	NaOH (0.1 M)	8.0g	Sigma S-5881
	H_2O, make up to	1000.0ml	
• Electrode buffer	Solution I	1600.0ml	
	Solution II	(until pH 7.4)	
• Gel buffer	Solution I	125.0ml	
	Solution II	(until pH 7.4)	
	H_2O, make up to	500.0ml	
C (St. Leger *et al.* 1992b):			
• Electrode buffer	Citric acid (0.04 M)	15.38g	Sigma C-7129
	N-(3-Aminopropyl)morpholine	20.0ml	Sigma A-9028
	H_2O, make up to	2000.0ml	
• Gel buffer	Citric acid (4 mM)	0.96g	Sigma C-7129
	N-(3-Aminopropyl)morpholine	1.25ml	Sigma A-9028
	H_2O, make up to	500.0ml	

Table 5 (cont.).

name	reagent	quantity	source
E (Ayala *et al.* 1973):			
• Electrode buffer	Tris (135 mM)	138.06g	Sigma T-1503
	Citric acid (45 mM)	65.96g	Sigma C-7129
	EDTA (1.2 mM)	0.74g	Sigma ED2SS
	H_2O, make up to	2000.0ml	
• Gel buffer	Tris (9 mM)	3.75g	Sigma T-1503
	Citric acid (3 mM)	1.44g	Sigma C-7129
	EDTA (1.2 mM)	0.24g	Sigma ED2SS
	H_2O, make up to	500.0ml	

Table 6. Staining protocols for the thirty-four enzymes used in this study. Refer to Table 8 for recommended name of enzyme corresponding to E.C. number given parenthetically below name.

Enzyme	reagent	quantity	source	notes
AAT	AAT buffer	50.0ml		(see Table 7)
(2.6.1.1)	Fast garnet GBC salt	1000.0mg	Sigma F-0875	
	Fast blue salt (optional)	200.0mg	Sigma F-025	
AC	Isocitrate dehydrogenase	100.0μl	Sigma I-2002	
(4.2.1.3)	Aconitic acid	75.0mg	Sigma A-7251	
	R gel buffer	80.0ml		(see Table 7)
	NADP	10.0mg	Sigma N-0505	(see glossary)
	MTT	10.0mg	Sigma M-2128	(see glossary)
	PMS	3.0mg	Sigma P-9625	(see glossary)
ADH	Ethanol	1.0ml		
(1.1.1.1)	R gel buffer	80.0ml		
	NAD	20.0mg	Sigma N-7004	(see glossary)
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	
AGA[1]	Citrate-A buffer	20.0ml		(see Table 7)
(3.2.1.53)	4-Methylumbelliferyl Acetamido-2-deoxy-β-D-galactopyranoside	5.0mg	Sigma M-9659	
	(Incubate gel at 37°C for 15 min., then spray on Glycine-NaOH)			(see Table 7)
AGP	α-Glycerophosphate	300.0mg	Sigma G-2138	
(1.1.1.8)	R gel buffer	80.0ml		
	NAD	20.0mg	Sigma N-7004	
AK	Glucose	200.0mg	Sigma G-8270	
(2.7.4.3)	ADP	100.0mg	Sigma A-0127	(see glossary)
	R gel buffer	80.0ml		
	G6PDH	60.0 units	Sigma G-7750	(see Table 8, 1.2.1.12)
	NADP	10.0mg	Sigma N-0505	
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	

Table 6 (cont.).

Enzyme	reagent	quantity	source	notes
CAR[1]	Phosphate-B buffer	50.0ml		(see Table 7)
(4.2.1.1)	Fluorescein diacetate (1% in acetone)	1.0ml	Sigma F-7378	
DIA	NADH	25.0mg	Sigma N-8129	(see glossary)
(1.8.1.4)	2,6-Dichlorophenolindophenol	2.0mg	Sigma D-1878	
	R gel buffer	80.0ml		
	MTT	10.0mg	Sigma M-2128	
EST	α-Naphthyl acetate	50.0mg	Sigma N-6875	
(3.1.1.1)	β-Naphthyl acetate	50.0mg	Sigma N-8505	
	Fast blue BB salt	50.0mg	Sigma F-0250	
	R gel buffer	80.0ml		
	(dissolve Naphthyl acetates in 1.0ml acetone).			
FDP	Fructose-1,6-diphosphate	40.0mg	Sigma 752-1	
(3.1.3.11)	PGI	100.0 units	Sigma P-9010	(see gloassary)
	R gel buffer	80.0ml		
	G6PDH	60.0 units	Sigma G-7750	
	NADP	10.0mg	Sigma N-0505	
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	
FUM	Fumaric acid	400.0mg	Sigma F-2752	
(4.2.1.2)	MDH	40.0μl	Sigma 410-13	(see Table 8, 1.1.1.37)
	R gel buffer	80.0ml		
	NAD	20.0mg	Sigma N-7004	
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	

Table 6 (cont.).

Enzyme	reagent	quantity	source	notes
G6PDH	Glucose-6-phosphate	400.0mg	Sigma G-7879	
(1.1.1.49)	R gel buffer	80.0ml		
	NADP	10.0mg	Sigma N-0505	
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	
GAPDH	Fructose-1,6-diphosphate	55.0mg	Sigma 752-1	
(1.2.1.12)	Aldolase	100.0 units	Sigma A-6253	
	R gel buffer	80.0ml		
	NAD	20.0mg	Sigma N-7004	
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	
GDH	Glutamic acid	2000.0mg	Sigma G-1626	
(1.4.1.2)	R gel buffer	80.0ml		
	NAD	20.0mg	Sigma N-7004	
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	
GK	Glucose	90.0mg	Sigma G-8270	
(2.7.1.2)	ATP	50.0mg	Sigma A-6521	(see glossary)
	R gel buffer	80.0ml		
	G6PDH	60.0 units	Sigma G-7750	
	NADP	10.0mg	Sigma N-0505	
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	
β-GLU	4-Methylumbelliferyl glucoside	75.0mg	Sigma M-3633	
(3.2.1.21)	R gel buffer	10.0ml		

Table 6 (cont.).

Enzyme	reagent	quantity	source	notes
GP	Glacial acetic acid	50.0ml	Fisher A38s-212	
(general)	H_2O	200.0ml		
	Ethanol, 95%	250.0ml	McCormick Distilling, Missouri	
	Nigrosin	250.0mg	Sigma N-4754	
	Buffalo black	250.0mg	Sigma N-3005	
GPI	Fructose-6-phosphate	50.0mg	Sigma F-3627	
(5.3.1.9)	R gel buffer	80.0ml		
	G6PDH	60.0 units	Sigma G-7750	
	NADP	10.0mg	Sigma N-0505	
	MTT	20.0mg	Sigma M-2128	
	PMS	6.0mg	Sigma P-9625	
GR	Glutathione (GSSG)	50.0mg	Sigma G-4626	
(1.6.4.2)	2,6-Dichlorophenolindolphenol	1.0mg	Sigma D-1878	
	R gel buffer	80.0ml		
	NADPH	20.0mg	Sigma N-7505	(see glossary)
	MTT	10.0mg	Sigma M-2128	
GUN[1]	Acetate-B buffer	40.0ml		(see Table 7)
(3.2.1.31)	(preincubate gel in this buffer for 15 min.)			
	4-Methylumbelliferyl-β-D-glucoronide	3.0mg	Sigma M-9130	
	Tris-E	20.0ml		(see Table 7)
	(Incubate gel at 37°C for 30 min., then spray on Glycine-NaOH)			(see Table 7)
HBDH	β-Hydroxybutyric acid	50.0mg	Sigma H-6501	
(1.1.1.30)	R gel buffer	80.0ml		
	NAD	20.0mg	Sigma N-7004	
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	

Table 6 (cont.).

Enzyme	reagent	quantity	source	notes
HEXA[1] (3.2.1.52)	4-Methylumbelliferyl-2-deoxy-β-D-glyopyranoside	5.0mg	Sigma M-2133	
	Citrate-A	20.0ml		(see Table 7)
HK (2.7.1.1)	Tris-B	10.0ml		(see Table 7)
	H_2O	90.0ml		
	$MgCl_2$ crystals	1000.0mg	Fisher M33-500	
	Glucose	200.0mg	Sigma G-8270	
	ATP	125.0mg	Sigma A-5394	
	G6PDH	60.0 units	Sigma G-7750	
	NADP	10.0mg	Sigma N-0505	
	MTT	20.0mg	Sigma M-2128	
	PMS	6.0mg	Sigma P-9625	
IDH (1.1.1.42)	Isocitrate	75.0mg	Sigma I-1252	
	$MgCl_2$ crystals	1000.0mg	Fisher M33-500	
	R gel buffer	80.0ml		
	NADP	10.0mg	Sigma N-0505	
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	
LDH (1.1.1.27)	Lactate (0.5 M)	10.0ml		(see Table 7)
	R gel buffer	80.0ml		
	NAD	20.0mg	Sigma N-7004	
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	
MADH (1.1.1.138)	Mannitol	100.0mg		
	R gel buffer	80.0ml		
	NADP	10.0mg	Sigma N-0505	
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	

Table 6 (cont.).

Enzyme	reagent	quantity	source	notes
MDH	Malic acid (pH 7.0)	10.0ml		(see Table 7)
(1.1.1.37)	R gel buffer	80.0ml		
	NAD	20.0mg	Sigma N-7004	
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	
ME	Malic acid (pH 7.0)	10.0ml		(see Table 7)
(1.1.1.40)	R gel buffer	80.0ml		
	NADP	10.0mg	Sigma N-0505	
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	
MPI	Mannose-6-phosphate	35.0mg	Sigma M-8754	
(5.3.1.8)	R gel buffer	80.0ml		
	G6PDH	60.0 units	Sigma G-7750	
	PGI	100.0 units	Sigma P-9010	
	NADP	10.0mg	Sigma N-0505	
	MTT	20.0mg	Sigma M-2128	
	PMS	6.0mg	Sigma P-9625	
PGM	Glucose-1-phosphate	100.0mg	Sigma G-7000	
(2.7.5.1)	R gel buffer	80.0ml		
	G6PDH	60.0 units	Sigma G-7750	
	NADP	10.0mg	Sigma N-0505	
	MTT	20.0mg	Sigma M-2128	
	PMS	6.0mg	Sigma P-9625	
SDH	Sorbitol	250.0mg	Sigma S-1876	
(1.1.1.14)	R gel buffer	80.0ml		
	NAD	20.0mg	Sigma N-7004	
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	

Table 6 (cont.).

Enzyme	reagent	quantity	source	notes
SKDH	Shikimic acid	100.0mg	Sigma S-5375	
(1.1.1.25)	R gel buffer	80.0ml		
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	
SOD	R gel buffer	80.0ml		
(1.15.1.1)	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	
XDH	Hypoxanthine	20.0mg	Sigma H-9377	
(1.1.1.204)	R gel buffer	80.0ml		
	NAD	20.0mg	Sigma N-7004	
	MTT	10.0mg	Sigma M-2128	
	PMS	3.0mg	Sigma P-9625	

1. Immediately observe fluorescent bands under ultraviolet light as the stain disappears rapidly.

Table 7. Buffers used in enzyme staining protocols. This table provides information regarding buffers cited in Table 6.

Buffer	alternate name	reagent	quantity	source
AAT		α-Ketoglutarate	0.75g	Sigma K-1750
		L-aspartate	2.75g	Sigma A-9006
		EDTA	1.00g	Sigma ED2SS
		PVP 40	10.0g	Sigma PVP-40
		NaH_2PO_4	15.0g	Sigma S-0751
		Na_2HPO_4	15.0g	Sigma S-0876
		H_2O, make up to	1000.0ml	
Acetate-B	Acetate/NaOH (0.15 M, pH 5.0)	Glacial acetic acid	9.3ml	Fisher A38s-212
		NaOH (in pellets)	5.1g	Sigma S-5881
		H_2O, make up to	900.0ml	
Citrate-A	Citrate/phosphate (0.1 M, pH 4.5)	Citric acid (0.1 M)	21.0g	Sigma C-7129
		H_2O, make up to	1000.0ml	
	(adjust to pH 4.5 with Na_2HPO_4 (1.0M)			
Glycine-NaOH	Glycine/NaOH (1.0 M, pH 10.0)	Glycine, free base	77.0g	Sigma G-7126
		H_2O, make up to	1000.0ml	
Lactate	Lactate (0.5 M)	Lactic acid	50.0ml	Sigma L-1250
		$NaHCO_3$	49.2g	Sigma S-8875
		H_2O, make up to	500.0ml	
Malic acid	Malic acid (pH 7.0)	D-L-Malic acid	33.5g	Sigma M-1000
		$NaHCO_3$	55.0g	Sigma S-8875
		H_2O, make up to	500.0ml	
Phosphate-B	Phosphate Na/Na_2 (0.01 M, pH 6.5)	NaH_2PO_4, 2 H_2O	9.98g	Sigma S-9638
		Na_2HPO_4 (anhydrous)	5.11g	Sigma S-0876

Table 7 (cont.).

Buffer	alt. name	reagent	quantity	source
R electrode buffer		Lithium hydroxide	2.52g	Sigma L-4256
		Boric acid	18.55g	Sigma B-0252
		H_2O, make up to	1.0l	
R gel buffer		Tris	72.6g	Sigma T-1503
		Citric acid	19.2g	Sigma C-7129
		R electrode buffer	200.0ml	(above)
		H_2O, make up to	20.0l	
Tris-A	Tris/HCl (0.2 M, pH 8.0)	EDTA	2.0g	Sigma ED2SS
		Tris	121.0g	(see glossary)
		H_2O, make up to	5000.0ml	
	(adjust to pH 8.0 with HCl).			
Tris-B	Tris (pH 8.5)	Tris-HCl (1.0 M)	2.21g	Sigma T-3253
		H_2O, make up to	50.0ml	

Table 8. The thirty-four enzymes used in the screening run; their recommended (E.C.) names, and corresponding buffer systems used in starch gel electrophoresis. See Table 6 (Appendix) for staining protocols of enzymes listed below.

E.C. Number[1]	Abbreviation	Recommended name	Buffer System[2]	Subunit Structure	Mode of detection[3]
Oxidoreductases					
1.1.1.1	ADH	Alcohol dehydrogenase	M	dimer	a
1.1.1.8	AGP	Glycerol-3-phosphate dehydrogenase (NAD^+)	M		
1.1.1.14	SDH	Sorbitol dehydrogenase			
1.1.1.25	SKDH	Shikimate dehydrogenase	M		
1.1.1.27	LDH	Lactate dehydrogenase	M, T, C	tetramer	b
1.1.1.30	HBDH	Hydroxybutyrate dehydrogenase	M		
1.1.1.37	MDH	Malate dehydrogenase	C, T	dimer	a
1.1.1.40	ME	Malic enzyme	C	tetramer	
1.1.1.42	IDH	Isocitrate dehydrogenase ($NADP^+$)	E		a
1.1.1.49	G6PDH	Glucose-6-phosphate dehydrogenase	M	dimer	a
1.1.1.138	MADH	Mannitol dehydrogenase			
1.1.1.204	XDH	Xanthine dehydrogenase	M		
1.2.1.12	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	M	tetramer	a
1.4.1.2	GDH	Glutamate dehydrogenase			a
1.6.4.2	GR	Glutathione reductase	M		
1.8.1.4	DIA	Dihydrolipoamide dehydrogenase	M		
1.15.1.1	SOD	Superoxide dismutase			
Transferases					
2.6.1.1	AAT	Aspartate amino-transferase			b, d
2.7.1.1	HK	Hexokinase	M	monomer	a
2.7.1.2	GK	Glucokinase	M		
2.7.4.3	AK	Adenylate kinase	M	monomer	
2.7.5.1	PGM	Phosphoglucomutase	E	monomer	

Table 8 (cont.).

E.C. Number[1]	Abbreviation	Recommended name	Buffer System[2]	Subunit Structure	Mode of detection[3]
Hydrolases					
3.1.1.1	EST	α- and β-Esterase	M, T		a
3.1.3.11	FDP	Fructose-biphosphatase	M		
3.2.1.21	β-GLU	β-Glucosidase (NAD(P)H)	M		c
3.2.1.31	GUN	β-Glucuronidase	M		c
3.2.1.52	HEXA	β-N-Acetylhexosaminidase	M		
3.2.1.53	AGA	β-N-Acetylgalactosaminidase	M		
Lyases					
4.2.1.1	CAR	Carbonate dehydratase	M		
4.2.1.2	FUM	Fumarate hydratase			
4.2.1.3	AC	Aconitate hydratase	M		
Isomerases					
5.3.1.8	MPI	Mannose-6-phosphate isomerase	C, T	monomer	
5.3.1.9	GPI	Glucose phosphate isomerase	M	dimer	
General protein	GP				

1. Numerical order according to the Nomenclature Committee of the International Union of Biochemistry (Webb 1984).
2. M = TM 7.4, T = TC 7.0 (see Materials and Methods, Starch Gel Electrophoresis, p. 18, and Table 2).
3. See Isozyme Analysis, Specificity of enzymatic reactions, p. 18:
   a. Simultaneous capture reaction,
   b. postincubation capture reaction,
   c. autochromic method,
   d. overlay method.

Table 9. Electromorphs of enzyme-buffer systems useful for distinguishing *P. farinosus* and *P. fumososroseus* (see Figure 1 for diagrams corresponding to phenotype numbers given in this table).

Isol.	AC	ADH	AGP	CAR	DIA	G6PDH	GP	GPI	GR	HBDH	MPI	PGM
PF601	1	2	1	1	1	3	2	3	2	1	2	1
PF602	2	2	2	4	1	2	2	2	2	1	2	1
PF603	2	2	2	4	1	2	2	2	2	1	2	1
PF604	2	2	2	4	1	2	2	2	2	1	2	1
PF606	2	2	-	4	-	2	2	n.t.	-	1	n.t.	n.t.
PF607	2	2	-	4	-	2	2	n.t.	-	1	n.t.	n.t.
PF608	-	2	-	4	-	-	2	n.t.	2	-	n.t.	n.t.
PF609	-	2	-	4	-	-	2	2	-	-	n.t.	n.t.
PFR600A	3	1	3	3	-	1	1	1	1	2	1	2
PFR601	3	1	3	3	-	1	1	1	1	2	1	2
PFR602	3	1	3	3	-	1	1	1	1	2	1	2
PFR603	3	1	3	3	-	1	1	1	1	2	1	2
PFR604	3	1	3	3	-	1	1	1	1	2	1	2
PFR605	-	1	3	3	-	1	1	n.t.	-	2	n.t.	n.t.
PFR606	3	1	3	n.t.	-	1	1	n.t.	-	2	n.t.	n.t.
PFR607	-	1	-	3	-	1	1	n.t.	-	2	n.t.	n.t.
PFR609	-	-	-	3	-	-	1	n.t.	-	2	n.t.	n.t.
PFR610	3	1	-	n.t.	-	1	1	1	-	2	n.t.	n.t.
PFR611	3	1	-	3	-	-	1	n.t.	-	-	n.t.	n.t.
PFR612	-	1	-	3	-	-	1	1	-	-	n.t.	n.t.
PFR613	3	1	-	n.t.	-	1	1	n.t.	-	2	n.t.	n.t.
PFR621	-	1	-	3	-	-	1	n.t.	-	2	n.t.	n.t.
PFR623	-	1	-	2	-	1	1	n.t.	-	-	n.t.	n.t.

- = no detectable enzymatic activity.
n.t. = not tested.

Table 10. Sample genetic diversity of each isolate for all thirty-nine loci. This table provides estimates of gene diversity measures to evaluate the significance of a difference between isolates. Calculations performed by GeneStat-PC (Lewis 1992). Enzymes (loci) are identified in the left column by their abbreviations listed in Tables 1, 6 (Appendix), and 8 (Appendix). Letter abbreviations across the top of the page refer to isolate code, Table 4 (Appendix).

	F1	s.e.	F2	s.e.	F3	s.e.	F4	s.e.
AAT	0.5000	0.0945	0.5000	0.0945	0.5000	0.0945	0.5000	0.0945
AC	******	******	******	******	******	0.0945	0.4800	0.0947
ADH-1	0.1723	0.0000	0.0000	0.0000	0.0000	0.0000	0.2451	0.1361
ADH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
AK	0.0000	0.0000	0.0000	0.0000	0.0000	0.1591	0.0000	0.0000
DIA	0.0000	0.0000	0.0000	0.0000	0.0000	0.1591	0.0000	0.0000
DIA	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
G6PDH-1	******	******	******	******	******	0.0000	0.0000	0.0000
G6PDH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0901	0.1421	0.0888
G6PDH-3	0.0945	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GAPDH	0.0000	0.0000	0.0000	0.0000	0.0000	0.1119	0.2451	0.1361
GK-1	******	******	******	******	******	0.1119	0.2978	0.1065
GK-2	******	******	******	******	******	0.0000	0.0000	0.0000
GP	******	******	******	******	******	0.0000	0.4442	0.1723
GPI-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GPI-2	******	******	******	******	******	0.1009	0.4875	0.0313
GPI-3	******	******	******	******	******	0.0636	0.2661	0.0816
GR	******	******	******	******	******	0.0478	0.0000	0.0000
HBDH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HBDH-2	0.1723	0.0000	0.0000	0.0000	0.0000	0.1723	0.0000	0.0000
HBDH-3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.3200	0.1591
IDH	0.1723	0.0000	0.0000	0.0000	0.0000	0.0945	0.0000	0.0000
LDH-1	******	******	******	******	******	0.1256	0.0000	0.0000
LDH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.1135	0.0000	0.0000
LDH-3	0.0000	0.0000	0.0000	0.0000	0.0000	0.1135	0.0000	0.0000
LDH-4	0.2041	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
MADH	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.5000	0.2041
MDH-1	0.2041	0.5000	0.2041	0.0000	0.0000	0.0761	0.4862	0.0444
MDH-2	******	******	******	******	******	0.0953	0.2782	0.1028
ME-1	******	******	******	******	******	0.0875	0.2782	0.1028
ME-2	******	******	******	******	******	0.1098	0.0000	0.0000
ME-3	******	******	******	******	******	0.1098	0.0000	0.0000
MPI-1	******	******	******	******	******	0.0000	0.0000	0.0000
MPI-2	******	******	******	******	******	0.0636	0.0000	0.0000
PGM	******	******	******	******	******	0.0000	0.0000	0.0000
SDH	******	******	******	******	******	0.0000	0.0000	0.0000
SOD	******	******	******	******	******	0.0000	0.0000	0.0000
XDH-1	******	******	******	******	******	0.0378	0.2978	0.1065
XDH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.1065	0.2978	0.1065
Means	0.1936	0.0305	0.2114	0.0336	0.2278	0.0330	0.1427	0.0301

Table 10 (cont.).

	F6	s.e.	F7	s.e.	F8	s.e.	F9	s.e.
AAT	******	******	******	******	******	******	******	******
AC	0.4442	0.1723	0.4442	0.1723	0.0000	0.0000	0.0000	0.0000
ADH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.4442	0.1723
ADH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
AK	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.5000	0.2041
DIA	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
DIA	******	******	******	******	******	******	0.0000	0.0000
G6PDH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
G6PDH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
G6PDH-3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GAPDH	******	******	******	******	******	******	0.0000	0.0000
GK-1	******	******	******	******	******	******	******	******
GK-2	******	******	******	******	******	******	******	******
GP	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GPI-1	******	******	******	******	******	******	0.0000	0.0000
GPI-2	******	******	******	******	******	******	0.0000	0.0000
GPI-3	******	******	******	******	******	******	0.0000	0.0000
GR	0.0000	0.0000	0.0000	0.0000	0.4442	0.1723	0.0000	0.0000
HBDH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HBDH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HBDH-3	0.0000	0.0000	0.4442	0.1723	0.0000	0.0000	0.0000	0.0000
IDH	******	******	******	******	******	******	******	******
LDH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
LDH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
LDH-3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
LDH-4	0.0000	0.0000	0.0000	0.0000	0.5000	0.2041	0.0000	0.0000
MADH	0.0000	0.0000	0.5000	0.2041	0.5000	0.2041	0.0000	0.0000
MDH-1	******	******	******	******	******	******	0.0000	0.0000
MDH-2	******	******	******	******	******	******	0.0000	0.0000
ME-1	******	******	******	******	******	******	0.0000	0.0000
ME-2	******	******	******	******	******	******	0.0000	0.0000
ME-3	******	******	******	******	******	******	0.0000	0.0000
MPI-1	******	******	******	******	******	******	******	******
MPI-2	******	******	******	******	******	******	******	******
PGM	******	******	******	******	******	******	******	******
SDH	******	******	******	******	******	******	******	******
SOD	******	******	******	******	******	******	******	******
XDH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
XDH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Means	0.0222	0.0222	0.0694	0.0380	0.0722	0.0395	0.0315	0.0219

Table 10 (cont.).

	R0	s.e.	R1	s.e.	R2	s.e.	R3	s.e.
AAT	0.5000	0.0945	0.3750	0.1687	0.5000	0.0945	0.5000	0.0945
AC	0.2451	0.1361	0.4442	0.1723	0.4800	0.0947	0.4800	0.0947
ADH-1	0.4937	0.0478	0.0000	0.0000	0.0000	0.0000	0.4937	0.0478
ADH-2	0.0000	0.0000	0.4800	0.0947	0.4800	0.0947	0.0000	0.0000
AK	0.0000	0.0000	0.3200	0.1591	0.3200	0.1591	0.3200	0.1591
DIA	0.2782	0.1474	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
DIA	0.3750	0.1135	0.5000	0.0945	0.5000	0.0945	0.4688	0.0741
G6PDH-1	0.0860	0.0569	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
G6PDH-2	0.4630	0.0422	0.4959	0.0378	0.4263	0.0734	0.4263	0.0734
G6PDH-3	0.0000	0.0000	0.0000	0.0000	0.2606	0.0990	0.0000	0.0000
GAPDH	0.5000	0.0257	0.4084	0.1119	0.4084	0.1119	0.2978	0.1065
GK-1	0.5000	0.0301	0.3454	0.1124	0.3454	0.1124	0.4937	0.0478
GK-2	0.2782	0.1028	0.3454	0.1124	0.1974	0.1163	0.1974	0.1163
GP	0.5000	0.0615	0.4442	0.1723	0.4442	0.1723	0.4442	0.1723
GPI-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GPI-2	0.4442	0.0420	0.4442	0.0613	0.4983	0.0233	0.4928	0.0220
GPI-3	0.0000	0.0000	0.2306	0.0917	0.2082	0.0850	0.1472	0.0646
GR	0.2978	0.1065	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HBDH-1	0.4084	0.1119	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HBDH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HBDH-3	0.0000	0.0000	0.4442	0.1723	0.4800	0.0947	0.4800	0.0947
IDH	0.0000	0.0000	0.4800	0.0947	0.4800	0.0947	0.0000	0.0000
LDH-1	0.2188	0.1256	0.3750	0.1135	0.3750	0.1135	0.3750	0.1135
LDH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
LDH-3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
LDH-4	0.0000	0.0000	0.3750	0.1135	0.3750	0.1135	0.0000	0.0000
MADH	0.0000	0.0000	0.5000	0.2041	0.5000	0.2041	0.2782	0.1474
MDH-1	0.0000	0.0000	0.3037	0.0871	0.3037	0.0871	0.4359	0.0460
MDH-2	0.0000	0.0000	0.1162	0.0747	0.1162	0.0747	0.0694	0.0462
ME-1	0.4688	0.0376	0.3200	0.1098	0.3200	0.1098	0.4630	0.0422
ME-2	0.0000	0.0000	0.1800	0.1081	0.3200	0.1098	0.0000	0.0000
ME-3	0.0000	0.0000	0.0000	0.0000	0.3200	0.1098	0.0000	0.0000
MPI-1	0.3200	0.1098	0.0000	0.0000	0.4084	0.1119	0.0000	0.0000
MPI-2	0.0000	0.0000	0.2451	0.1361	0.0000	0.0000	0.0000	0.0000
PGM	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
SDH	0.5000	0.0945	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
SOD	0.0000	0.0000	0.3750	0.1687	0.0000	0.0000	0.0000	0.0000
XDH-1	0.4800	0.0425	0.3969	0.0901	0.3969	0.0901	0.1800	0.1081
XDH-2	0.2306	0.0917	0.4630	0.0636	0.3969	0.0901	0.3200	0.1098
Means	0.1946	0.0339	0.2412	0.0321	0.2528	0.0320	0.1888	0.0335

Table 10 (cont.).

	R4	s.e.	R5	s.e.	R6	s.e.	R7	s.e.
AAT	0.5000	0.0945	******	******	******	******	******	******
AC	0.4442	0.1723	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ADH-1	0.4800	0.0947	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ADH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
AK	0.3200	0.1591	******	******	0.0000	0.0000	0.0000	0.0000
DIA	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
DIA	0.0000	0.0000	******	******	******	******	******	******
G6PDH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
G6PDH-2	0.4688	0.0741	0.5000	0.2041	0.0000	0.0000	0.5000	0.2041
G6PDH-3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GAPDH	0.4442	0.1061	******	******	******	******	******	******
GK-1	0.5000	0.0456	******	******	******	******	******	******
GK-2	0.0000	0.0000	******	******	******	******	******	******
GP	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GPI-1	0.0000	0.0000	******	******	******	******	******	******
GPI-2	0.4800	0.0425	******	******	******	******	******	******
GPI-3	0.2306	0.0917	******	******	******	******	******	******
GR	0.4084	0.1119	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HBDH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HBDH-2	0.4442	0.1723	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HBDH-3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
IDH	0.0000	0.0000	******	******	******	******	******	******
LDH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
LDH-2	0.3750	0.1135	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
LDH-3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.5000	0.2041
LDH-4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
MADH	0.5000	0.2041	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
MDH-1	0.3037	0.0871	******	******	******	******	******	******
MDH-2	0.0000	0.0000	******	******	******	******	******	******
ME-1	0.3200	0.1098	******	******	******	******	******	******
ME-2	0.0000	0.0000	******	******	******	******	******	******
ME-3	0.0000	0.0000	******	******	******	******	******	******
MPI-1	0.4084	0.1119	******	******	******	******	******	******
MPI-2	0.0000	0.0000	******	******	******	******	******	******
PGM	0.5000	0.0945	******	******	******	******	******	******
SDH	0.0000	0.0000	******	******	******	******	******	******
SOD	0.0000	0.0000	******	******	******	******	******	******
XDH-1	0.1974	0.1163	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
XDH-2	0.1974	0.1163	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Means	0.1929	0.0341	0.0263	0.0263	0.0000	0.0000	0.0500	0.0344

Table 10 (cont.).

	R9	s.e.	R10	s.e.	R11	s.e.	R12	s.e.
AAT	******	******	******	******	******	******	******	******
AC	0.0000	0.0000	0.4442	0.1723	0.0000	0.0000	0.0000	0.0000
ADH-1	0.0000	0.0000	0.4442	0.1723	0.0000	0.0000	0.3200	0.1591
ADH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
AK	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
DIA	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
DIA	******	******	0.5000	0.2041	******	******	0.0000	0.0000
G6PDH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
G6PDH-2	0.0000	0.0000	0.5000	0.0945	0.0000	0.0000	0.0000	0.0000
G6PDH-3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GAPDH	******	******	0.0000	0.0000	******	******	0.0000	0.0000
GK-1	******	******	******	******	******	******	******	******
GK-2	******	******	******	******	******	******	******	******
GP	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GPI-1	******	******	0.5000	0.0945	******	******	0.5000	0.0456
GPI-2	******	******	0.0000	0.0000	******	******	0.0000	0.0000
GPI-3	******	******	0.0000	0.0000	******	******	0.0000	0.0000
GR	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HBDH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HBDH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HBDH-3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
IDH	******	******	******	******	******	******	******	******
LDH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
LDH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
LDH-3	0.0000	0.0000	0.0000	0.0000	0.5000	0.2041	0.0000	0.0000
LDH-4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
MADH	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
MDH-1	******	******	0.5000	0.0615	******	******	0.5000	0.0301
MDH-2	******	******	0.0000	0.0000	******	******	0.0000	0.0000
ME-1	******	******	0.5000	0.0615	******	******	0.5000	0.0301
ME-2	******	******	0.0000	0.0000	******	******	0.0000	0.0000
ME-3	******	******	0.0000	0.0000	******	******	0.0000	0.0000
MPI-1	******	******	******	******	******	******	******	******
MPI-2	******	******	******	******	******	******	******	******
PGM	******	******	******	******	******	******	******	******
SDH	******	******	******	******	******	******	******	******
SOD	******	******	******	******	******	******	******	******
XDH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
XDH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Means	0.0000	0.0000	0.1129	0.0381	0.0250	0.0250	0.0607	0.0292

Table 10 (cont.).

	R13	s.e.	R21	s.e.	R23	s.e.
AAT	******	******	******	******	******	******
AC	0.4442	0.1723	0.0000	0.0000	0.0000	0.0000
ADH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
ADH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
AK	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
DIA	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
DIA	******	******	******	******	******	******
G6PDH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
G6PDH-2	0.5000	0.0945	0.0000	0.0000	0.0000	0.0000
G6PDH-3	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GAPDH	******	******	******	******	******	******
GK-1	******	******	******	******	******	******
GK-2	******	******	******	******	******	******
GP	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
GPI-1	******	******	******	******	******	******
GPI-2	******	******	******	******	******	******
GPI-3	******	******	******	******	******	******
GR	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HBDH-1	0.4442	0.1723	0.0000	0.0000	0.0000	0.0000
HBDH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
HBDH-3	0.4442	0.1723	0.0000	0.0000	0.0000	0.0000
IDH	******	******	******	******	******	******
LDH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
LDH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
LDH-3	0.5000	0.2041	0.0000	0.0000	0.0000	0.0000
LDH-4	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
MADH	0.5000	0.2041	0.5000	0.2041	0.0000	0.0000
MDH-1	******	******	******	******	******	******
MDH-2	******	******	******	******	******	******
ME-1	******	******	******	******	******	******
ME-2	******	******	******	******	******	******
ME-3	******	******	******	******	******	******
MPI-1	******	******	******	******	******	******
MPI-2	******	******	******	******	******	******
PGM	******	******	******	******	******	******
SDH	******	******	******	******	******	******
SOD	******	******	******	******	******	******
XDH-1	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
XDH-2	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Means	0.1416	0.0498	0.0250	0.0250	0.0000	0.0000

Table 11. Nei's Genetic Identities, *I* (Above) and Distances, *D* (Below); and Means of Identities (right) and Distances (below).

	F1	F2	F3	F4	F6	F7	F8	F9
F1		0.9186	0.8494	0.8692	0.7664	0.7408	0.7644	0.8891
F2	0.0849		0.9701	0.9506	0.8737	0.8531	0.8529	0.8635
F3	0.1633	0.0304		0.9107	0.8777	0.8564	0.8473	0.8424
F4	0.1402	0.0506	0.0936		0.9454	0.9646	0.9396	0.9219
F6	0.2661	0.1350	0.1304	0.0561		0.9929	0.9580	0.9496
F7	0.3001	0.1589	0.1550	0.0361	0.0072		0.9707	0.9353
F8	0.2687	0.1591	0.1657	0.0623	0.0429	0.0297		0.9478
F9	0.1175	0.1467	0.1715	0.0813	0.0517	0.0669	0.0536	
R0	0.1856	0.0683	0.1495	0.0809	0.0645	0.0842	0.1363	0.1614
R1	0.1533	0.0867	0.1033	0.0270	0.1268	0.0875	0.1471	0.1076
R2	0.1281	0.0739	0.1056	0.0140	0.1050	0.0776	0.0997	0.0842
R3	0.1125	0.0569	0.1043	0.0060	0.0355	0.0275	0.0629	0.0646
R4	0.1681	0.0845	0.1384	0.0422	0.1207	0.1106	0.1428	0.1644
R5	0.2016	0.1493	0.1490	0.0667	0.1503	0.1273	0.1558	0.0694
R6	0.2337	0.2584	0.2191	0.1775	0.2522	0.2314	0.2611	0.1218
R7	0.1985	0.2000	0.1830	0.1381	0.2177	0.1969	0.2267	0.0873
R9	0.1979	0.1883	0.1842	0.1146	0.1890	0.1675	0.1952	0.0652
R10	0.2700	0.1645	0.1378	0.1274	0.0647	0.0810	0.1211	0.1841
R11	0.2607	0.1646	0.1436	0.0743	0.0297	0.0431	0.0302	0.0391
R12	0.2228	0.1586	0.1522	0.1229	0.0755	0.0907	0.0775	0.1529
R13	0.2786	0.1792	0.1871	0.0837	0.0494	0.0242	0.0779	0.1170
R21	0.3505	0.2342	0.2310	0.0803	0.0842	0.0339	0.0581	0.0952
R23	0.2495	0.1485	0.1439	0.0619	0.0204	0.0326	0.0200	0.0292
Means	0.2069	0.1355	0.1474	0.0790	0.1034	0.0986	0.1179	0.1015

Table 11 (cont.).

	R0	R1	R2	R3	R4	R5	R6	R7
F1	0.8306	0.8578	0.8798	0.8936	0.8453	0.8174	0.7916	0.8199
F2	0.9340	0.9170	0.9287	0.9447	0.9190	0.8613	0.7723	0.8187
F3	0.8612	0.9018	0.8998	0.9010	0.8708	0.8615	0.8032	0.8328
F4	0.9223	0.9734	0.9861	0.9940	0.9586	0.9355	0.8373	0.8711
F6	0.9376	0.8809	0.9003	0.9651	0.8863	0.8605	0.7771	0.8043
F7	0.9193	0.9162	0.9253	0.9728	0.8953	0.8805	0.7935	0.8213
F8	0.8726	0.8633	0.9051	0.9391	0.8669	0.8558	0.7702	0.7972
F9	0.8509	0.8980	0.9193	0.9375	0.8484	0.9330	0.8853	0.9164
R0		0.9212	0.9360	0.9496	0.9403	0.8636	0.7374	0.8000
R1	0.0821		1.0000	0.9851	0.9289	0.9451	0.8405	0.8976
R2	0.0662	0.0000		0.9911	0.9417	0.9454	0.8491	0.8976
R3	0.0517	0.0150	0.0089		0.9565	0.9587	0.8649	0.9131
R4	0.0616	0.0738	0.0601	0.0445		0.8152	0.7543	0.7735
R5	0.1466	0.0565	0.0562	0.0422	0.2043		0.9376	1.0000
R6	0.3046	0.1737	0.1636	0.1452	0.2819	0.0644		0.9316
R7	0.2232	0.1081	0.1080	0.0909	0.2568	0.0000	0.0709	
R9	0.2317	0.1065	0.0967	0.0823	0.2451	0.0088	0.0513	0.0168
R10	0.1058	0.1549	0.1417	0.0958	0.1691	0.1625	0.3022	0.1670
R11	0.1217	0.1732	0.1261	0.0539	0.1651	0.1361	0.2379	0.1717
R12	0.1603	0.1882	0.1508	0.0969	0.1704	0.1017	0.1419	0.1074
R13	0.0753	0.1209	0.1212	0.0622	0.1490	0.1506	0.2968	0.1909
R21	0.1879	0.1159	0.1060	0.0559	0.1996	0.0755	0.1754	0.1409
R23	0.1071	0.1554	0.1097	0.0412	0.1487	0.1232	0.2231	0.1886
Means	0.1298	0.1074	0.0911	0.0617	0.1455	0.1090	0.1995	0.1495

Table 11 (cont.).

	R9	R10	R11	R12	R13	R21	R23	Means
F1	0.8205	0.7634	0.7705	0.8002	0.7568	0.7044	0.7792	0.8149
F2	0.8284	0.8483	0.8482	0.8533	0.8360	0.7912	0.8620	0.8748
F3	0.8317	0.8713	0.8663	0.8588	0.8294	0.7938	0.8660	0.8638
F4	0.8918	0.8804	0.9284	0.8844	0.9197	0.9228	0.9400	0.9249
F6	0.8278	0.9374	0.9708	0.9273	0.9518	0.9192	0.9798	0.9041
F7	0.8458	0.9222	0.9578	0.9133	0.9761	0.9667	0.9679	0.9085
F8	0.8227	0.8860	0.9703	0.9255	0.9251	0.9436	0.9802	0.8911
F9	0.9369	0.8318	0.9616	0.8582	0.8896	0.9092	0.9712	0.9044
R0	0.7932	0.8996	0.8854	0.8519	0.9275	0.8287	0.8984	0.8801
R1	0.8990	0.8565	0.8410	0.8284	0.8861	0.8906	0.8561	0.8993
R2	0.9078	0.8679	0.8815	0.8600	0.8859	0.8995	0.8961	0.9138
R3	0.9210	0.9086	0.9475	0.9077	0.9397	0.9456	0.9597	0.9408
R4	0.7826	0.8444	0.8478	0.8433	0.8616	0.8190	0.8618	0.8664
R5	0.9912	0.8501	0.8727	0.9033	0.8602	0.9273	0.8841	0.8982
R6	0.9500	0.7392	0.7882	0.8677	0.7432	0.8391	0.8000	0.8215
R7	0.9833	0.8462	0.8422	0.8982	0.8263	0.8686	0.8281	0.8631
R9		0.7914	0.8391	0.9182	0.7982	0.8900	0.8500	0.8691
R10	0.2340		0.9553	0.9039	0.9608	0.8492	0.9132	0.8694
R11	0.1754	0.0457		0.9390	0.9426	0.9310	0.9917	0.8990
R12	0.0853	0.1011	0.0629		0.8684	0.8877	0.9485	0.8840
R13	0.2253	0.0399	0.0591	0.1411		0.9239	0.9267	0.8834
R21	0.1166	0.1635	0.0715	0.1191	0.0792		0.9408	0.8814
R23	0.1625	0.0908	0.0084	0.0529	0.0761	0.0610		0.9046
Means	0.1427	0.1420	0.1088	0.1242	0.1266	0.1289	0.1025	