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
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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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APPROVAL

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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

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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).