# 7 Monitoring the Fate of Biocontrol Fungi

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## Introduction

The release of any endemic, exotic or genetically modified microbe into the ecosystem generates some apprehension in the general public and the scientific community. This apprehension revolves around the conceivable negative effects on human health and ecosystem stability. We are caught in a dilemma where, on the one hand, we desire to manipulate certain parts of our natural or agricultural ecosystem through these microbial releases and, on the other, we wish to maintain ecosystem stability and to reduce risks to human health.

Microorganisms have been released regularly into the environment over the last 15 years and this has resulted in some successes. This includes the inoculation of *Rhizobium* spp. into the soil for enhancement of nitrogen fixation, the application of several *Pseudomonas* species for bioremediation and the inoculation of fungi for biological control of agricultural pests and noxious weeds (Julien, 1987). In 1987, the first environmental introduction of a lacZY-engineered strain of *Pseudomonas aureofaciens* (L11) was approved by the US Environmental Protection Agency (Kluepfel *et al.*, 1991). Although it may appear paradoxical, the production, release and study of a genetically modified organism may yet provide the best insight into aspects of microbial ecology since the modified strain can be tracked in the environment. L11 was tracked over three successive crop rotations and provided some valuable information on the population dynamics (i.e. persistence and fate) of bacteria in the rhizosphere (Kluepfel, 1993).

The release of biocontrol fungi is sometimes perceived as a threat to human welfare and environmental stability. The North American Microbial Biocontrol Working Group considered several possible environmental and human health effects associated with the release of microorganisms for pest control. These were: (i) the competitive displacement of non-target organisms; (ii) human allergens; (iii) toxicity to non-target organisms; and (iv) pathogenicity to non-target organisms (Cook *et al.*, 1996). Risks associated with the release of fungal biocontrol agents are discussed in more detail in Goettel and Jaronski (1997). In order to alleviate public and scientific concerns with fungal introduction, it is evident that the risk evaluation agency or individuals

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(eds T.M. Butt, C. Jackson and N. Magan)

releasing a biocontrol agent must also have the means to track and identify it in the environment. To date, there is little information available on the fate of a single fungal clone released into the environment nor is there any information available on the transfer of genes from released fungi to other individuals in the population or to other species.

## Biocontrol Fungi and the 'New' Green Revolution

There are several features of biocontrol fungi which garner public support. First and foremost, they may potentially displace or reduce the application of chemical products, many of which are toxic to humans and the environment. Biocontrol products currently hold less than 2% of the global pesticide market but this was not always the case. Biocontrol agents were used before chemical pesticides were developed. For example, the first recorded use of an insect-pathogenic fungus was the application of *Metarhizium anisopliae* to control the wheat cockchafer, *Anisoplia austriaca*, in Ukraine by Metschnikoff in 1879 (Ferron, 1978). *M. anisopliae* was also used to control the sugar-cane froghopper, *Aeneolamia saccharina*, in Trinidad at the turn of the century (Rorer, 1913). *Erynia radicans* was employed against aphids in apple orchards in Canada in the 1920s (Dustan, 1924).

At the onset of the 1950s, and the 'green revolution', the slogan 'better living through chemistry' shifted the pest control focus from a biocontrol approach to a chemical approach, which was easier, faster-acting and more effective. However, the benefits of chemical pesticide application are typically based only on direct crop returns. They do not include the indirect environmental, economic and human health costs associated with pesticides (Pimentel *et al.*, 1992). The application of a variety of chemicals over the last 40 years has left the agriculture and forestry industries with pesticide resistance in pests, environmental toxicity, fishery losses, groundwater and surface-water contamination, depletion of rhizosphere microorganisms and human health concerns.

In the 1980s the effort to return to biological control was met with some successes. Many examples are found for fungal biocontrol agents. A classical biocontrol approach was used in the application of *Zoophthora radicans* to control spotted alfalfa aphid in Australia. The fungus quickly spread from the point of introduction and was successful in control of the aphid (Milner *et al.*, 1982). In Europe, success in controlling the cockchafer, *Melolontha melolontha*, using *Beauveria brongniartii* was achieved (Keller, 1989). In Australia, *M. anisopliae* has been formulated to control a major scarab pest, *Aphodius tasmanae*, a pest of pasture grasses (Roberts and Hajek, 1992). *M. anisopliae* has also been used to control sugar-cane pests in Brazil for more than 15 years (Moscardi, 1989). In China, *Beauveria bassiana* has been applied on a large scale to control pine-moth larvae (Xu, 1988). Success has also been achieved with plant biocontrol fungi. Massion and Lindow (1986) have shown control of Canada thistle with *Puccinia obtegens*, yellow nutsedge with *Puccinia canaliculata* and Johnson grass with *Sphacelotheca holci*. Daniel *et al.* (1973) successfully controlled northern joint-vetch, *Aeschynomene virginia*, in a rice crop with *Colletotrichum gloeosporioides* f. sp. *aeschynomene.*

*Biocontrol fungi provide an alternative to chemical pesticides but caution should be exercised in their release; do we really understand fungal epidemiology?*

The application of biocontrol fungi has also met with many failures, though these are not widely reported in the scientific literature. As an example, much research had been done in England, Canada and Australia on the mycoparasite, *Coniothymium minitans* for biological control of the plant–pathogenic *Sclerotinia* species but each research group discontinued the project, based primarily on the poor performance of *C. minitans* in the field (Adams, 1990). Large quantities of the fungal propagules were required when applied directly to the soil, where the mycoparasites were met by microbial antagonists.

## Measuring Fungal Biocontrol Success

One of the major setbacks with biocontrol has been its inconsistent record (compared with the relative consistency of chemical control). It has been almost impossible to identify reasons for the failures or, for that matter, the successes of fungal biological control introductions. This is partially due to an inadequate understanding of the epidemiology and ecology of these fungi in the field. Improving our understanding of disease epizootiology is critical to understanding the successes or failures of fungal biocontrol agents and also to the understanding of fungal microbial ecology. As an integral part of the measurement of success or failure of a biocontrol fungus, one has to ask the following questions. What was the fate of the released fungus? Was it really the released fungus that caused the desired effect? The answers can only be ascertained with an adequate understanding of the genetic structure of the endemic fungal population and a method of identifying and monitoring the persistence and fate of the introduced fungal clone.

> *The ability to monitor a biocontrol fungal release has three major uses; the first is the identification of the introduced agent in a biological impact assessment; secondly, it allows for a rational economic cost/benefit analysis of the fungal release; and, thirdly, it provides valuable information about fungal epidemiology.*

The following review consists of three major sections. First, I shall introduce the relevance of the need to identify the introduced fungal biocontrol agents as part of an environmental risk assessment. Secondly, I shall cursorily review molecular and nonmolecular methods available for identifying the genetic variability in biocontrol fungi. Finally, I shall present a case-study of the release of an exotic fungal pathogen of insects into the prairie ecosystem.

## Risk Assessment in Biocontrol Releases

Biological impact assessment is a process designed to evaluate the possible risks of releasing specific endemic or exotic organisms. The application or augmentation of biocontrol fungi is designed to have a specific effect on the ecosystem. It may even be argued that the release of a biocontrol fungus is meant to engender change in the targeted system. The risk evaluation asks whether the researcher can predictably meet the demands of agriculture and at the same time preserve the ecological integrity of the impact area in a sustainable manner.

Risk assessments have been conducted with a variety of fungal biocontrol agents of weeds. In 1975 the first permit for field testing of the fungal plant pathogen,

*C. gloeosporioides* (now registered as Collego), was issued as a biocontrol agent for weed control. This field release was issued only after a 6-year test period in a containment which showed that the field release would be safe (Templeton, 1975). Toxicity to mammals and survival of the fungus in the field were studied. The fate and survival of the fungus in non-target aquatic organisms have also been evaluated (Genthner *et al.*, 1993).

*Public perception and scientific calculation of the potential impact of biocontrol releases motivate political conviction to fund assessment impact programmes.*

An integral part of a biological impact assessment is that some type of marker be employed to monitor and detect the microorganism introduced into the environment. This microorganism should be monitored in the environment against a background of the microbial community into which it is introduced. In particular, it should be possible to distinguish the introduced microorganism from those related to it in the microbial community.

## Methods of Identifying the Genetic Individual

The term genetic individual is used in plant biology (Harper, 1977) and more recently in fungi (Rayner, 1991) and is sometimes referred to as the genet. The genet includes all clonally reproducing individuals of a mitotic cell lineage that originates from an individual. Risk assessment studies require that the genet be tracked in the field against the background fungal population. Tracking the genet would also provide an idea of the efficacy of the biocontrol agent, patterns of cycling of the biocontrol agent in the environment and dispersal and persistence in the soil. Simply releasing a fungus into the environment and evaluating the desired impact lack the precision of identifying the genet in an epizootiological investigation.

Most of the fungal biocontrol releases to date compare the effects of a fungal release on the host insect or plant population at the release site with that of a control site. This is fraught with problems since the same species of biocontrol fungi may be found naturally at both the release and the control sites and it may be impossible to distinguish the resident population from the introduced genet. It would also be impossible to determine the synergistic effects of the resident fungal population with that of the introduced genet. For example, the insect-pathogenic fungi *M. anisopliae* or *B. bassiana*, or the plant-pathogenic fungi *Colletotrichum* spp. are ubiquitous (Sutton, 1992; Bidochka *et al.*, 1998). There must be some means by which the particular genet that has been chosen for the application can be positively identified and distinguished from the background population in the field.

*After the release of a biocontrol fungus, the causal relationship between a specific biocontrol fungus and a specific disease will require genetic characterization of the endemic population and comparison with the introduced genet.*

In the past, fungal taxonomists have relied on phenotypic markers, such as fungal and conidial colony characteristics, vegetative compatibility and mating type, to differentiate individuals. These markers may be useful in differentiating species or certain groups within species, but they generally lack the resolution to distinguish a genet within a population. Deficiencies in traditional microbial detection techniques have led to research into new methodologies and, in particular, molecular techniques. These

techniques have the sensitivity and selectivity required to track fungal genets released into the environment. Lessons in monitoring biocontrol fungi can probably best be learned from the vast number of techniques employed to track bacteria released into the environment (Prosser, 1994). The techniques fall into four major groups: morphological methods, immunological methods, nucleic acid probing and the introduction of molecular markers, or 'tagging'.

The major problem with utilizing DNA-based techniques for identifying the fungal genet is that they provide information on the presence and perhaps total cell concentrations but they do not provide information on the viability or activity of the introduced organisms. The introduction of molecular markers is designed to confer a distinctive genotype/phenotype on the transformed fungus, which enables tracking in the environment. The advantage is that this method provides information on the viability and activity of the introduced organism, but it also has its drawbacks. Special care must be taken to avoid introduction of the gene marker (tag) within any genomic DNA region that may cause genetic instability, impair fitness or affect, in any way, expression of metabolic regulatory genes. The expression of the marker system could impair the fitness of the organism if an undue metabolic burden is imposed by the expression and maintenance of the marker gene.

Several excellent reviews are available on methods of determining genetic variability in fungi, such as those by Bruns *et al.* (1991), Burdon (1993), Leslie (1993), McDermott and McDonald (1993) and Rosewich and McDonald (1994). There are many fungi that are being commercially exploited, as outlined in Table 7.1, and there will be a growing necessity to monitor these releases. Due to the large number of examples of biocontrol fungi, the following section will focus on methods used to identify genets in a plant biocontrol fungus, *C. gloeosporioides* (see also Manners and He, 1997), and several species of deuteromycetous (*M. anisopliae* and *B, bassiana*) and zygomycetous insect-pathogenic fungi. However, many of these techniques can be applied to just about any fungi mentioned in Table 7.1. The suitable utility (and associated problems) of applying techniques for monitoring in epizootiological studies is outlined in Table 7.2.

## *Morphological and biochemical markers and vegetative compatibility groups (VCGs)*

Morphological markers are restricted to those pathogens that are easily cultured on artificial media. Many morphological and antibiotic-resistance variants have been identified in biocontrol fungi. These include colony variants in mycelial or conidial colour, growth rate, colony surface texture, topology and viscosity and resistance to antibiotics. Morphological variants and antibiotic resistance may occur as spontaneous mutants or may be generated through mutagenesis. For example, acriflavine-resistant mutants occur spontaneously in *M. anisopliae* at a mutation rate as high as $1 \times 10^{-9}$ (Tinline and Noviello, 1971). The major problem associated with the use of morphological or antibiotic-resistance mutants is that they are unstable and may revert back to a wild type. Colony morphology also varies considerably in many fungal species and this morphology may not be stable upon subculturing since many fungi exhibit pleiomorphic deterioration of colony characteristics. Reasons for phenotypic instability in fungi include changeable chromosomal complements, transposons, cytoplasmically transmitted genetic elements (e.g. cytoplasmic RNAs and invertrons) and DNA

**Table 7.1.** A selection of fungal species and how they are being commercially exploited.

| Fungal species | Biological activity | Application |
|---|---|---|
| *Ampelomyces quisqualis* | Biological fungicide | Germinating spores suppress the development of powdery mildews on a variety of crops (e.g cucumbers, apples, grapes, ornamentals, strawberries and tomatoes) by hyperparasitism |
| *Beauveria bassiana* | Biological insecticide | Invades insect body and kills the host insect. Target pests include European corn-borer, Asiatic corn-borer, whiteflies, thrips, aphids and mealy bugs. Target crops include maize, vegetables and ornamentals |
| *Beauveria brongniartii* | Biological insecticide | Target pests include white grubs and cockchafers infesting sugar cane and barley |
| *Candida oleophila* | Biological fungicide | Prevents postharvest diseases in citrus fruits by producing secondary metabolites inhibitory to storage-disease fungi |
| *Chondostereum purpureum* | Biological herbicide | A wood-rot fungus that invades cut stumps on target trees such as American black cherry, yellow birch and poplar |
| *Colletotrichum gloeosporioides* f. sp. *aechynomene* | Biological herbicide | Plant pathogen with specificity to the weed, northern joint-vetch |
| *Coniothyrium minitans* | Biological fungicide | A non-pathogenic fungus that grows on leaf surfaces and prevents the invasion of plant pathogens such as *Sclerotinia* |
| *Endothia parasitica* | Biological fungicide | A non-pathogenic strain of this fungus that grows on potential infection sites on trees and prevents the invasion of pathogens |
| *Fusarium oxysporum* | Biological fungicide | A non-pathogenic strain of this fungus that protects crops from pathogenic strains of *Fusarium* by competing for sites at the root-infection sites |
| *Gliocladium catenulatum* | Biological fungicide | Produces secondary metabolites inhibitory to some plant pathogens, such as *Pythium* spp. and *Rhizoctonia* spp. |
| *Metarhizium anisopliae* | Biological insecticide | Insect pathogen used on a variety of crops, including greenhouse vegetables and ornamentals |
| *Myrothecium verrucaria* | Biological nematocide | Fungal mycelia invade plant-parasitic nematodes present in the soil |
| *Paecilomyces fumosoroseus* | Biological insecticide | For the control of whitefly, aphids, thrips and spider mites |
| *Phlebiopsis gigantea* | Biological fungicide | Competes for entry sites and prevents establishment of the rot fungus, *Heterobasidum annosum*, on pine and spruce stumps |
| *Phytophthora palmivora* | Biological herbicide | A specific pathogen of the roots of the strangler vine or milkweed vine |

**Table 7.1.** continued

| Fungal species | Biological activity | Application |
|---|---|---|
| *Pythium oligandrum* | Biological fungicide | Outcompetes pathogenic soil fungi in greenhouse crops, outdoor vegetables and cereal crops |
| *Trichoderma harzianum* | Biological fungicide | Competes for soil nutrients with plant-pathogenic fungi such as *Botrytis* and *Sclerotinia* |
| *Verticillium lecanii* | Biological insecticide | Used primarily for the control of whitefly and aphids in greenhouses |

**Table 7.2.** Methods used to identify population level variability in fungi and their adequacy or potential for monitoring introduced clones into the ecosystem. The level of natural variation observed in a population is dependent on fungal species and certain demes. The relative qualitative assessment of low to high variation is a general estimate based on studies to date.

| | Level of natural variation observed | | |
|---|---|---|---|
| Method | Low | High | Adequacy as a marker system |
| Colony morphology | ------> | | Unstable, subject to reversion |
| Derived mutants, e.g. antibiotic resistance, conditional mutants | --> | | Unstable, subject to reversion. May have reduced fitness (e.g. a conditional mutant), which may be desirable. Potential for antibiotic resistance transfer into natural populations |
| Karyotype analysis | ------> | | Technique is lengthy (up to 3 days for a CHEF) |
| Allozymes | --------> | | Easy to score and identify if introduced clone has unique alleles. Fungus must be easy to culture since a relatively large amount of material is required |
| dsRNA | ----------> | | dsRNA isolation can be difficult. Fidelity is suspect. Transmission of dsRNA to other isolates possible |
| Transposable elements | ------->(?) | | Identification difficult. Few examples |
| RAPD | -----------------> | | Technique is relatively easy. Small quantities of DNA required. Fungus does not need to be cultured. Potential problems with reproducibility |
| RFLP | ---------------> | | Technique involves relatively large quantities of DNA. Appropriate probe required |
| Directed PCR | ---------------> | | Technique is relatively easy. Small quantities of DNA required. Pathogen does not require culturing. Some difficulty in screening for variability at the population level |
| DNA probes | --------------->(?) | | Same as directed PCR |
| VCG | --------> | | Vegetative compatability studies in some fungi requires the production of complementation mutants and a large number of comparisons |
| Introduced molecular markers | NA | | Unique in the population. Ease of transformation of many fungi. Stability of markers still questionable. Effects on fungal fitness questionable |

CHEF, clamped homogeneous electrical field; dsRNA, double-stranded RNA; NA, not applicable; RAPD, random amplification of polymorphic DNA; RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction; VCG, vegetative compatability groups.

and RNA polymerases that may act as mitochondrial retroposons (Kistler and Miao, 1992).

Two populations of *C. gloeosporioides* from citrus differed in mycelial colour, growth rate, serine esterase profiles, restriction fragment length polymorphism (RFLP) of ribosomal DNA (rDNA) and Southern hybridization of a cutinase gene (Liyanage *et al.*, 1992, 1993). Variations in colony parameters were observed, such as patterns of concentric conidial production in response to light and darkness, perithecia and conidial production, conidial size and appressorium production (Menezes and Hanlin, 1996). Kuramae-Izioka *et al.* (1997) showed variation in *Colletotrichum* spp. (*Colletotrichum acutatum* vs. *C. gloeosporioides*) based on mycelial growth, benomyl resistance, pathogenicity and random amplification of polymorphic DNA (RAPD) patterns.

Another approach to identifying plants or insects infected by a certain fungus would be to utilize differences in sterol and fatty acid profiles of fungi (Mueller *et al.*, 1994). Although this approach would be a good initial diagnosis of fungal identification, its utility in identifying a fungal genet within a species has yet to be shown.

Conditional lethal mutants (heat-sensitive mutants) have been generated using ultraviolet (UV) mutagenesis in *B. bassiana* (Hegedus and Khachatourians, 1994). Heat-sensitive mutants grew at 20°C but not 30°C. Not only could such mutants be used to monitor pathogenicity development in an infected insect but they could also be used to limit the stability of and/or detect in the environment a conditional lethal strain. The use of such strains could be of some utility in situations, such as inundative applications, where cycling of the pathogen in the ecosystem is undesirable.

VCGs have been frequently used in plant pathology to evaluate fungal population structure. However, many fungi are anamorphic (asexual), precluding any studies of sexual compatibility. One of the methods used to investigate compatibility reactions, occuring either through heterokaryosis or parasexual recombination, is to select chlorate-resistant mutants and then test for complementary nitrate reductase, *nia* and *cnx*, mutants. Chlorate conversion to toxic chlorite is presumably catalysed through one of the nitrate-reducing enzymes. Thus, chlorate resistance may be due to a mutation at one of those loci. Couteaudier and Viaud (1997) analysed VCGs in *B. bassiana* using this method and observed that the VCGs correlated to RFLP patterns using a telomeric probe. VCGs could limit gene flow, resulting in demic subdivisions in the fungal population. Similarly, isolates within each biotype of *C. gloeosporioides* that infects legumes (*Stylosanthes* spp.; biotypes A and B) were vegetatively compatible but it was not possible to form heterokaryons between biotypes (Masel *et al.*, 1996). Selectable markers (hygromycin and phleomycin resistance) carried on vectors containing telomeric sequences from *Fusarium oxysporum* were used to transform isolates of the biotypes (Poplawski *et al.*, 1997). These were then allowed to contact each other, which resulted in a double-antibiotic-resistant progeny, but these were slow-growing.

VCGs may be considered good markers for monitoring biocontrol fungi if the introduced biotype has a distinctive VCG that is incompatible with individuals in the endemic population.

## *Immunological markers*

Immunological markers have been primarily used to distinguish species differences in fungi. Immunological markers and, in particular, enzyme-linked immunosorbent assays (ELISA) have been reported for the detection of many plant-pathogenic fungi (Schotts

*et al.*, 1994). Serological tests have also been used to identify *B. bassiana, M. anisopliae* and species of the Entomophthorales (Fargues *et al.*, 1981; Shimizu and Aizawa, 1988; Guy and Rath, 1990). A potential problem in employing ELISA is the ability of the polyclonal antibody to react with genetically similar species. For example, Hajek *et al.* (1991a) developed an ELISA with polyclonal antibodies to the plasma membrane of *Entomophaga maimaiga*, a pathogen of gypsy-moth larvae, *Lymantria dispar*. However, a closely related fungus, *Entomophaga aulicae*, showed cross-reactivity with the antisera.

## Allozymes, karyotypes and molecular markers

### Allozymes

Electrophoretic separation of allozymes was one of the first techniques used to screen for genetic variability in fungal populations. Conventionally, what is required is between 8 and 20 allozymes, depending on the degree of polymorphism and the number of alleles at each locus. Evaluation of 120 isolates of *M. anisopliae* showed that they fell into 48 distinctive genotypic classes. A large amount of variability was found for *M. anisopliae*, with less than 20% similarity among some classes (St Leger *et al.*, 1992a). Similar results were found for *B. bassiana* (St Leger *et al.*, 1992b). Allozyme analysis has also been applied in an attempt to distinguish pathotypes of *B. brongniartii*. Isolates showed high intraspecific variation and little association was observed between genotypic class and virulence to European cockchafer (*M. melolontha*) larvae (Reineke and Zebitz, 1996).

If the fungal strain that is being released into the field is distinctive at several loci it is sufficient to use allozyme analysis for identifying the genet. Allozymic variation has been used to monitor an entomophthoralean fungal genet in several studies. For example, Silvie *et al.* (1990) used allozyme analysis to show the fate and survival of a strain of *Pandora neoaphidis* released to control greenhouse aphids. The released strain was eventually displaced by endemic strains. Milner and Mahon (1985) showed that allozyme analysis could be used to distinguish an Israeli isolate of *Z. radicans* from isolates endemic to Australia. The Israeli isolate was released and provided control in populations of spotted alfalfa aphids.

### Karyotype analysis

One approach to identifying the genet has been to look at chromosomal length polymorphism (CLP), using pulsed-field gel electrophoresis (PFGE) for identifying fungal karyotypes. The most commonly employed PFGE technique is clamped homogeneous electrical field (CHEF). This technique has been used in several insect-pathogenic and plant-pathogenic fungi and the results show fungal genome sizes between 30 and 45 Mb and the number of chromosomes varies between five and nine. The chromosomes range in size from 0.2 to 10 Mb. Natural populations of fungi have a high degree of CLP (Mills and McCluskey, 1990), particularly the small, 200–600 kb, 'minichromosomes'.

In *C. gloeosporioides*, the minichromosomes were highly variable between isolates. DNA additions or deletions were also associated with the CLP and differences were observed within and between each biotype (Masel *et al.*, 1993). Analysis showed that

some genes found on a 1.2 Mb chromosome were unique and that high-copy-number repeat sequences were not present, suggesting that the genome of the progenitor strain underwent a large-scale deletion or addition from a genetically distinct strain. Interestingly, with a background of relatively high CLP variation within each biotype, there was little variation observed using RFLP analysis (He *et al.*, 1996).

CLPs have been observed in the insect-pathogenic fungus *B. bassiana* (Viaud *et al.*, 1996). Although there was some variability between strains, this variability was not great enough to identify genets. The CLPs observed were also correlated with telomeric, RFLP and RAPD markers. Shimizu *et al.* (1992) identified the electrophoretic karyotype in five isolates of *M. anisopliae*. Chromosomal sizes were between 1.6 and 7.2 Mbp and the total genome size between 29 and 33 Mbp. The five *M. anisopliae* isolates could be readily distinguished from one another.

Although extremely useful for genomic analysis, the drawback to this karyotyping method is that the procedure is lengthy and not necessarily discriminatory among strains. Furthermore, CLP may vary during subculturing fungi in the laboratory (e.g. *Magnaporthe grisea* (Talbot *et al.*, 1993)). It seems unlikely that CLP can be used as a technique by itself to identify the genet. However, when combined with other techniques it may be used to identify and map the positions of specific genes on various chromosomes.

### Molecular markers

Base substitutions, such as insertions or deletions, in the genome can be detected with RFLP. What one requires is a probe that will identify size variations in certain parts of the digested genomic DNA. Probes that have been used to detect inter- and intraspecific variation in fungi are rDNA (Pipe *et al.*, 1995), mitochondrial DNA (mtDNA) (Hegedus and Khachatourians, 1993a) and telomeric DNA (Couteaudier and Viaud, 1997).

Cloned DNA probes are portions of the genomic DNA identified through various subtractive or differentiation screening methods that hybridize to a particular strain or species of fungus. Kosir *et al.* (1991) used a cloned DNA probe to differentiate a strain of *B. bassiana* virulent to grasshoppers, *Melanoplus sanguinipes*, from a less virulent strain. Hegedus and Khachatourians (1993b) developed DNA probes that could differentiate *B. bassiana* from several other deuteromycetous entomopathogenic fungi.

An excellent example of the utility of molecular probes in a fungal epidemiological study has been the investigation of an entomophthoralean pathogen of gypsy-moth larvae, *L. dispar.* Gypsy-moth larval populations in the eastern USA were frequently infected by an entomophthoralean fungus. Hajek and co-workers utilized RFLP analysis, allozymes and cloned DNA probes to differentiate two closely related entomophthoralean fungi, *E. aulicae* and *E. maimaiga* (Soper *et al.*, 1983; Walsh *et al.*, 1990). By applying these molecular techniques to infected gypsy-moth larvae collected in the field, it was determined that *E. maimaiga* was the infectious fungus in these epizootics (Hajek *et al.*, 1996a, b, c). This in itself was interesting since *E. maimaiga* and gypsy-moth larvae are both exotic species to North America (Hajek *et al.*, 1995). This study is one of the few long-term epidemiological studies where a biocontrol fungus, *E. maimaiga*, has been identified and tracked using molecular probes (Hajek *et al.*, 1990, 1991a; Walsh *et al.*, 1990). The information gathered also allowed for the augmentative introduction of *E. maimaiga* to control gypsy moth at the leading edge of their expanding range (Hajek *et al.*, 1990). The probes have also been used to track

fungal persistence and dispersal and to evaluate physiological versus ecological host ranges of the fungus (Hajek *et al.*, 1996a).

RAPD utilizes the polymerase chain reaction (PCR) and single primers of between 15 and 20 nucleotides in length in order to detect variability in arbitrary regions of the genome. The technique is powerful due to its relative speed and the large number of loci that can be screened. Problems associated with RAPDs include the appearance and disappearance of minor bands with different runs and variability between thermocyclers from different manufacturers (Ellsworth *et al.*, 1993). However, taking this into consideration, the appearance or absence of the major bands is a quick and easy method to identify a genet. Using RAPD analysis, wide genetic variation in isolates of *M. anisopliae* was observed on a worldwide level (Fegan *et al.*, 1993; Bidochka *et al.*, 1994; Tigano-Milani *et al.*, 1995). An RAPD study of Brazilian isolates of *M. anisopliae* by Fungaro *et al.* (1996) showed that there was large variability of soil isolates (47% similarity) compared with those isolates from a hemipteran insect (*Deois flavopicta*) host (82% similarity).

RAPD markers have been used in several epidemiological studies in entomophthoralean fungi. In 1990 and 1991 seven isolates of *Z. radicans* originating from Serbia were released into a lucerne plot in New York state, USA, to control potato leafhopper, *Empoasca fabae* (Wraight *et al.*, 1986). RAPDs were used to distinguish released isolates of *Z. radicans* from North American isolates and indicated that the Serbian isolates had successfully established at the experimental release sites (Hodge *et al.*, 1995).

*Zoophthora phytonomi* in North America is a pathogen of the clover-leaf weevil, *Hypera punctata*. In 1973, *Z. phytonomi* was first noted to infect alfalfa weevil, *Hypera postica*, a species introduced from Eurasia (Hajek *et al.*, 1996c). RAPD patterns distinguished two major genotypic classes of *Z. phytonomi*; one genotype was principally isolated from *H. postica*. One explanation is that one of the genotypic classes was introduced from Eurasia, perhaps with the introduced alfalfa weevil, and subsequently spread.

Amplified restriction length polymorphisms (AFLP) have been used to detect genetic variation in plant-pathogenic fungi (Majer *et al.*, 1996). It is considered to be a reliable, reproducible technique that evaluates genomic variation at ten to 100 times more sites than either RAPDs or RFLP.

Targeted PCR refers to the amplification and analysis of particular regions of the genome, either by direct sequencing or RFLP analysis of the amplified region. Neuveglise and Brygoo (1994) showed that 28S rDNA differed in size between two strains of *B. brongniartii*. Curran *et al.* (1994) investigated the internal transcribed spacer (ITS) regions and 5.8S of *Metarhizium* rDNA. Primers for targeted PCR were developed from a specific cloned DNA probe in order to differentiate *B. bassiana* from other entomopathogenic fungi (Hegedus and Khachatourians, 1995). Ribosomal DNA sequences were used to elucidate the relationships within the genus *Colletotrichum* from New Zealand fruit-rotting isolates (Johnston and Jones, 1997) and biotypes A and B infecting legumes, *Stylosanthes* spp., in Australia (Braithwaite *et al.*, 1990). ITS regions of 18 species of *Colletotrichum* were used to gain an understanding of the phylogeny and systematics within the genus (Sreenivasaprasad *et al.*, 1996). These studies show the evolutionary relationships of various strains or may be used in a broad taxonomic application for identifying a fungal species. However, they have not been shown as useful in identifying the genet. One targeted PCR-based approach for identifying genets within insect- and plant-pathogenic fungi would be to fingerprint fungi based on

microsatellite variation (Bridge *et al.*, 1997a). Identifying species-specific microsatellite markers is potentially time-consuming; however, their utility in identifying a fungal genet is effective.

If certain amplified regions are subjected to further analysis, the information may be used to identify the fungal genet. RFLP analysis of the rDNA in *M. anisopliae* showed that some strains could be differentiated according to their geographic origins (Pipe *et al.*, 1995). RFLP analysis of the *Pr1* (extracellular protease-encoding) gene amplified from various isolates of *M. anisopliae* showed a correlation of RFLP profiles with geographic origin (Leal *et al.*, 1997). Because of the spatial distribution related to rDNA or *Pr1* RFLP, these marker systems could be applied to detect an *M. anisopliae* isolate released into another geographic location.

Another approach to identifying genets of *M. anisopliae* was to utilize a variety of biochemical and molecular techniques (Bridge *et al.*, 1997a). Isozyme analysis, RAPD and protease production were used to investigate the relationships between 30 isolates of *M. anisopliae*. Similarly, an approach that utilized biochemical markers and a phylogenetic approach based on ITS nucleotide differences was used to differentiate various species and isolates within the genus *Verticillium* (Bidochka *et al.*, 1999). However, identification of a fungal genet based on several techniques is not as efficient as a single discriminating technique.

In some cases, RFLP analysis is used to discriminate among fungal pathotypes. *B. brongniartii* was analysed by RFLP analysis of ITS regions amplified by PCR (Neuveglise *et al.*, 1994) and allowed separation of the strains into distinctive subgroupings. One of the subgroupings contained strains virulent to the white grub (*Hopochelus marginalis*). Variability in the ITS regions was due to point mutations, which occurred as frequently as 14.7% and 16.7% in the ITS1 and ITS2 regions, respectively. Ribosomal DNA and mtDNA variation in *C. gloeosporioides* showed an association with geographic origin or host (avocado, banana or papaya), except for the isolates from mango (Hodson *et al.*, 1993). RAPD markers showed similar results. A mango biotype of *C. gloeosporioides* was differentiated from eight other isolates from different fruit species (Hayden *et al.*, 1994). Pathogenicity bioassays showed that isolates pathogenic to mango were specifically highly virulent to mango, while the other isolates displayed a wide host range. The isolates from mango had the same rDNA and similar mtDNA RFLP patterns, regardless of geographic origin (eastern or western hemisphere).

A method for rapidly screening a large number of isolates of *B. bassiana* and *Aspergillus niger* has recently been developed, based on direct PCR from colonies treated with Novozym 234, a powder produced from the extracellular enzymes from *Trichoderma harzianum* (van Zeijl *et al.*, 1998). This could allow for rapid screening of a large number of fungal isolates for identification after a biocontrol release.

### *Double-stranded RNA viruses and transposable elements*

Double-stranded RNA (dsRNA) viruses are found in a wide range of fungal species (Dickinson, 1986). There was some anticipation that dsRNAs could act as markers in population studies (Burdon, 1992). However, this seems unlikely since their fidelity as markers is suspect and dsRNA transfer between fungal strains or species is not fully understood in many species of biocontrol fungi. In natural populations of insect-pathogenic fungi in Canada, dsRNAs were found in 38% and 17% of the *M. anisopliae* and *B. bassiana* isolates, respectively (Melzer and Bidochka, 1998). This study com-

pared dsRNA banding patterns from insect pathogenic fungi found in Canada with fungi (*M. anisopliae, B. bassiana* and *Metarhizium flavoviride*) found from areas as diverse as Oman, Trinidad, Benin, Mali and Australia. Double-stranded RNA banding patterns could be distinctive for an isolate or could be shared among species from diverse geographic origins. Similarly, dsRNAs may not be faithfully transmitted during subculturing of the fungus (Melzer and Bidochka, 1998). In some cases, the presence of dsRNAs is associated with reduced fitness of the pathogen (e.g. *Cryphonectria parasitica*; van Alfen *et al.*, 1975). Double-stranded RNA is conceivably transferable and is not a good indication of the genetic relationships between strains.

A transposon-like element termed *Cg*T1, unique to biotype B but not biotype A of *C. gloeosporioides*, was identified and found to contain motifs homologous to gag-like proteins, reverse transcriptase and ribonuclease (RNAse) H domains of the non-long-terminal-repeat, LINE-like class of retrotransposons (He *et al.*, 1996). PCR primers designed to amplify *Cg*T1 could be used to distinguish biotype A from biotype B. Maurer *et al.* (1997) isolated a transposable element, named *hupfer*, from *B. bassiana* by trapping it in the nitrate reductase structural gene. The transposable element had an open reading frame similar to the IS630- or *mariner-Tc*1-like transposases. Although transposable elements are common in fungi, the variability and stability of these elements is not well understood. Therefore, at this time, their utility as genetic markers is unknown.

## Introduced molecular markers

Transformation of fungi is now a fairly routine procedure. However, the first requirement for transformation with a marker gene is that the expressed phenotype should not be exhibited in the indigenous population. For example, the presence of bacteria with β-glucuronidase activity limits the application of β-glucuronidase (GUS) marker systems (Jefferson, 1989). In fungi, this is not a problem. Stable GUS co-transformants of *M. anisopliae* have been produced by either electroporation or using the gene gun (St Leger *et al.,* 1995). *M. anisopliae* has also been transformed with benomyl resistance (Bernier *et al.*, 1989; Goettel *et al.*, 1989; Bogo *et al.*, 1996; Valadares-Inglis and Inglis, 1997). The difficulty with many of the insect-pathogenic fungi, such as *M. anisopliae*, is that they are resistant to antibiotics, such as hygromycin (R.J. St Leger, personal communication), for which resistance genes are available. There are also environmental concerns regarding the release of organisms with antibiotic-resistant genes.

Other options for tagging include transformation with genes encoding green fluorescent protein or luminometric markers, such as the *lux*AB gene sequences, particularly if their expression is controlled by an inducible promoter (Shaw *et al.*, 1992). Some markers affect the competitive ability of the pathogen and are therefore less useful for monitoring to predict survival. However, with an inducible promoter the introduced genes would not generally be expressed in the fungus. The most important advance provided by marker systems is the ability to measure cell activity without the requirement for extraction of cells or the need to culture organisms.

## A Case-study in Monitoring a Fungal Biocontrol Release: Release of an Australian Fungal Pathogen of Grasshoppers in North America

The following is a case-study of the release of an exotic fungus to control grasshoppers in the USA. I briefly outline the fungal biology, the release programme, the public and scientific concerns raised with the release, the problems encountered with identifying the fungal genet and, finally, the outcome of the study.

### *The fungal pathotypes*

*Entomophaga grylli* is a zygomycetous fungal pathogen of grasshoppers that has a worldwide distribution. Much of the work on the life-history patterns, host ranges and ecology of *E. grylli* has been carried out in conjuction with the US Department of Agriculture Agricultural Research Service (USDA-ARS) and primarily by R.I. Carruthers, M.E. Ramos, D.L. Hostetter, W.A. Ramoska and R.S. Soper. At least three pathotypes have been identified within this 'species complex', which show differences in life cycle, host range and isozyme patterns (Carruthers *et al.*, 1997). Two pathotypes (pathotypes 1 and 2) have been identified as native to North America and pathotype 3 is native to Australia. Several other genetically distinctive strains (pathotypes?) have been identified from Japan and the Philippines (Walsh *et al.*, unpublished); however, little is known of their host ranges and biology.

Pathotype 1 infects members of the grasshopper subfamily Oedipodinae, the band-winged grasshoppers. The most serious grasshopper pest in this subfamily is *Camnula pellucida*, a pest on rangeland and adjacent crops. Pathotype 1 is also characterized by the production of resting spores within the grasshopper cadaver and aerial conidia. Given adequate moisture, the fungus is able to sporulate from the intersegmental membranes of the grasshopper. These conidia may then horizontally infect other grasshoppers. Resting spores lie dormant in the soil until suitable conditions for germination occur (Carruthers *et al.*, 1997).

Pathotype 2 differs from pathotype 1 in several respects. First, it preferentially infects members of the grasshopper subfamily Melanoplinae, the spur-throated grasshoppers. A species in this subfamily is *M. sanguinipes*, a periodically serious pest on cereal crops. Pathotype 2 is characterized by the inability to produce aerial conidia and it produces resting spores in the grasshopper cadaver. Pathotype 2 also produces cryptoconidia but horizontal transmission by these spores is considered to be minimal (Carruthers *et al.*, 1997).

Pathotype 3 contains the combined characteristics of pathotypes 1 and 2. It was isolated from a cyrtacanthracridine grasshopper in Australia (Milner, 1978). In the laboratory it exhibited a broader host range and infected North American oedipodine and melanopline grasshoppers (Ramoska *et al.*, 1988). Pathotype 3 sporulated more rapidly than pathotype 1, thus allowing for horizontal transmission, but it also produces resting spores, which can lie dormant.

### *The biocontrol strategy*

Although fungal epizootics in North America by one endemic pathotype of *E. grylli* may have caused a significant impact on one group of grasshoppers (Reigert, 1968),

the epizootic may be benign to another group of grasshoppers. In 1987, after the worst outbreak of grasshoppers in 50 years, the USDA Animal and Plant Health Inspection Service (APHIS) concluded that years of chemical control were not only environmentally harmful but chemical control was simply not working. Biological control of grasshoppers was deemed an alternative. Based on the recommendation of the Intraagency Grasshopper Integrated Pest Management committee, the implementation of pathotype 3 was determined to be a feasible tactic for the management of rangeland grasshopper populations. North American grasshopper species have had no contact with the Australian fungal pathogen and, theoretically, have not evolved resistance mechanisms to that pathogen. When an exotic pathogen is introduced, one expects the targeted host insect to be vulnerable.

From 1989 to 1991, approval was granted for the release of pathotype-3-infected grasshoppers (*Melanoplus differentialis*) at two field sites in McKenzie County, North Dakota. Approximately 5000 pathotype-3-infected grasshoppers were released by USDA-APHIS during the 3-year period at the two sites (Ramos, 1993). After the release, a population model showed that pathotype 3 was having an impact on grasshopper numbers (Carruthers *et al.*, 1997). However, it was also determined that *E. grylli* pathotypes 1 and 2 were also present at the release sites.

## *The controversy*

In 1993 a 'News and Comments' article (Goodman, 1993) appeared in the journal *Science* outlining the negative impact that pathotype 3 could have on native non-target grasshopper species and the prairie ecosystem as a whole. Theoretical problems associated with the establishment of an exotic fungal pathotype were noted in articles authored by Lockwood (1993a, b). Lockwood speculated that, if the pest grasshopper species declined, density-dependent mechanisms could result in pathotype 3 infecting non-target grasshopper species. Low-density grasshopper species, such as *Melanoplus femurrubrum*, an intermediate host of a parasite of several bird species, or *Hesperotettix viridis*, a 'beneficial' grasshopper species that feeds on the noxious weed, snakeweed, could be affected. Concerns over the movement of pathotype 3 from the inoculation area were also raised. Infected grasshoppers still not debilitated by fungal infection could carry pathotype 3 substantial distances in 1 day.

The long-term fate, dispersal and environmental impact of pathotype 3 were entirely at question, since an adequate method of differentiating the three pathotypes from one another had not been resolved. Resting spores are indistinguishable among the pathotypes. A method of positively and easily identifying pathotypes of this obligate grasshopper pathogen was required.

## *Identifying the fungal pathotypes*

*E. grylli* pathotypes had previously been shown to differ in allozyme banding patterns (Soper *et al.*, 1983). This technique required that the fungal protoplasts be grown in complex media (Grace's insect medium supplement with 10% (v/v) fetal bovine serum) from germinated resting spores or viable conidia obtained from infected grasshoppers (MacLeod *et al.*, 1980). Germination frequency of resting spores and conidia in the laboratory is very low (Stoy *et al.*, 1988) and the procedure is tedious. Once an

appropriate biomass had been obtained, the pathotypes could be differentiated based on allozyme patterns. For an obligate pathogen such as *E. grylli*, the application of this technique is logistically problematic for the evaluation of several hundred or 1000 infected grasshoppers. *E. grylli* pathotypes have also been shown to differ in rDNA polymorphisms based on RFLP analysis with a *Saccharomyces cerevisiae* rDNA probe (Walsh *et al.*, 1990). But here again relatively large amounts of good-quality DNA (*c.* 5–10 μg) need to be recovered from each infected grasshopper cadaver.

The criteria that needed to be filled in order for the technique to be suitable for a large-scale screening programme were that: (i) the technique must differentiate among the three pathotypes; (ii) only small amounts of DNA must be required; (iii) the technique must be amenable to screening large numbers of grasshoppers (of the order of 1000 infected grasshoppers); and (iv) the technique must be sensitive to *E. grylli* DNA extracted from resting spores residing in the grasshopper. We investigated three techniques in order to differentiate the three pathotypes (Bidochka *et al.*, 1995, 1997).

The first technique utilized RAPD technology to differentiate among the three pathotypes. The DNA from each pathotype that was used as the template was obtained from culture-collection isolates of the three pathotypes reared as protoplasts on Grace's insect culture media. RAPD banding patterns were specific for each pathotype and little variability was observed among strains within a pathotype (Bidochka *et al.,* 1995). However, a major obstacle was the extraction of DNA from resting spores residing in the infected grasshopper. Resting spores have thick cell walls and are recalcitrant to enzymatic or chemical digestion. The resting spores could be physically disrupted using a homogenizer. However, the DNA isolated from physically disrupted resting spores showed some degradation, possible because of the physical action of the homogenizer. When RAPD-PCR was attempted using this DNA as the template, the results were ambiguous and inconsistent.

The second technique we employed was to cut out specific bands produced by the RAPD-PCR and use these as probes against DNA extracted from *E. grylli* resting spores. Results showed pathotype-specific probes; however, the probes showed only moderate to very light signal strengths.

Finally, *Hin*dIII-digested DNA fragments from genomic DNA of the three pathotypes were cloned and screened for pathotype specificity. Figure 7.1 outlines the strategy used to clone pathotype-specific probes that showed good signal strengths. Further analysis of these probes showed that they were moderately or highly repetitive in the fungal genome. The probes also contained AT-rich regions and contained repeats of several different simple sequence motifs (Walsh *et al.*, unpublished).

### *Application of molecular probes to field-collected grasshoppers*

Live grasshoppers were collected by sweep-netting every 7–10 days at the North Dakota release sites and areas concentric to the release sites during the summers of 1992 to 1994. The grasshoppers were identified to species, instar and adult sex and then brought to indoor facilities, where they were housed. Grasshopper mortality was checked daily and the abdomen dissected and examined for the presence of resting spores, using a microscope (Ramos, 1993). The infected grasshoppers were kept frozen (–20°C) until the application of the pathotype-specific probes.

In the laboratory, the infected grasshoppers were homogenized in order to fracture the resting spores and the DNA was extracted. DNA was dot-blotted on to nitro-

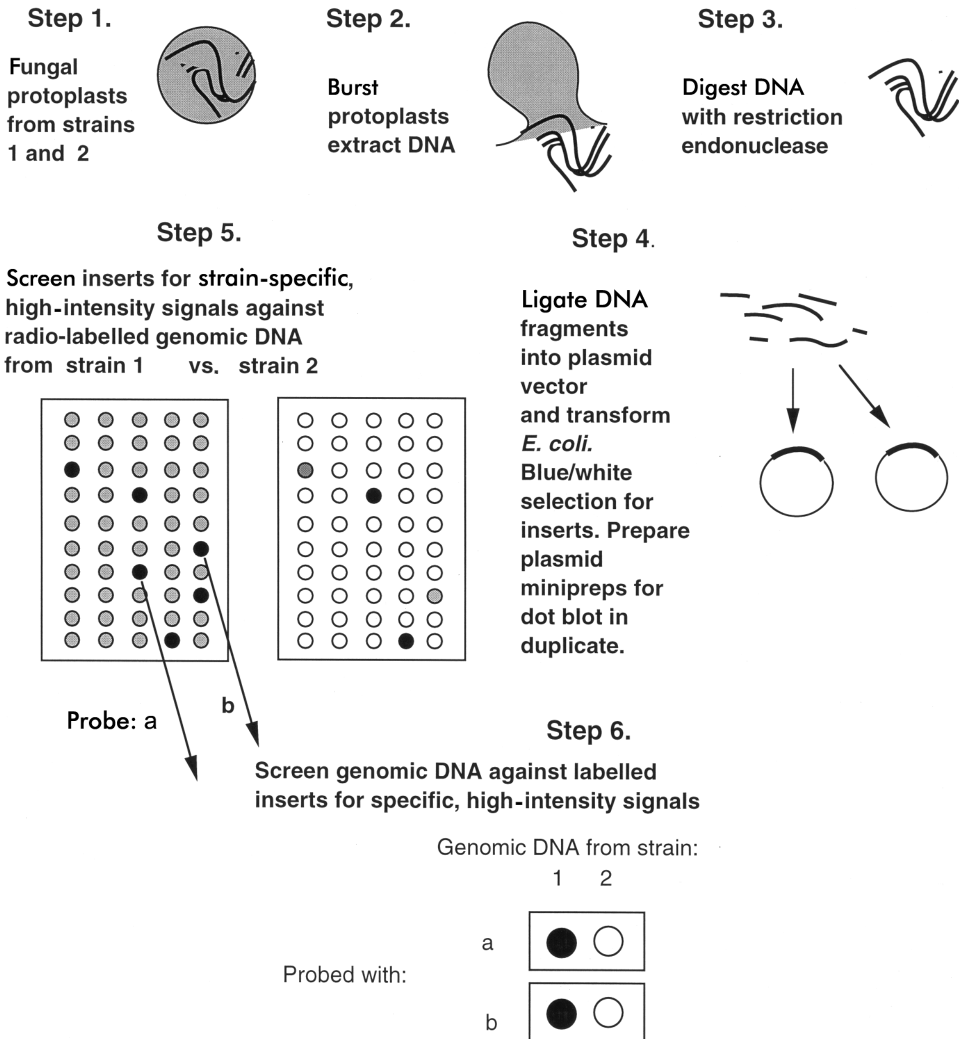


**Fig. 7.1.** The strategy used to develop a specific DNA probe to distinguish, in this example, strain 1 from strain 2. This strategy would be repeated for each strain for which you require a probe. The same strategy was employed to develop specific DNA probes to distinguish *Entomophaga grylli* pathotypes 1, 2 and 3. In this case, since three pathotypes were involved, three membranes would be used for hybridization against genomic DNA from a separate pathotype or genomic DNA from two pathotypes would be combined for the subtractive comparison on one membrane.

cellulose membranes in triplicate and each membrane was hybridized with a radiolabelled pathotype-specific DNA probe. Figure 7.2 shows a representative autoradiograph of 24 grasshoppers analysed for pathotype-specific infection. In total, 1216 grasshoppers were analysed (888 within 1 km of the release sites and 243 concentric to the release sites). Of the infected grasshoppers collected and analysed, 92.7% showed positive signals for one or more of the three pathotypes (Bidochka *et al.*, 1996). The percentage of *E. grylli*-infected grasshoppers at the release sites declined over 3 years

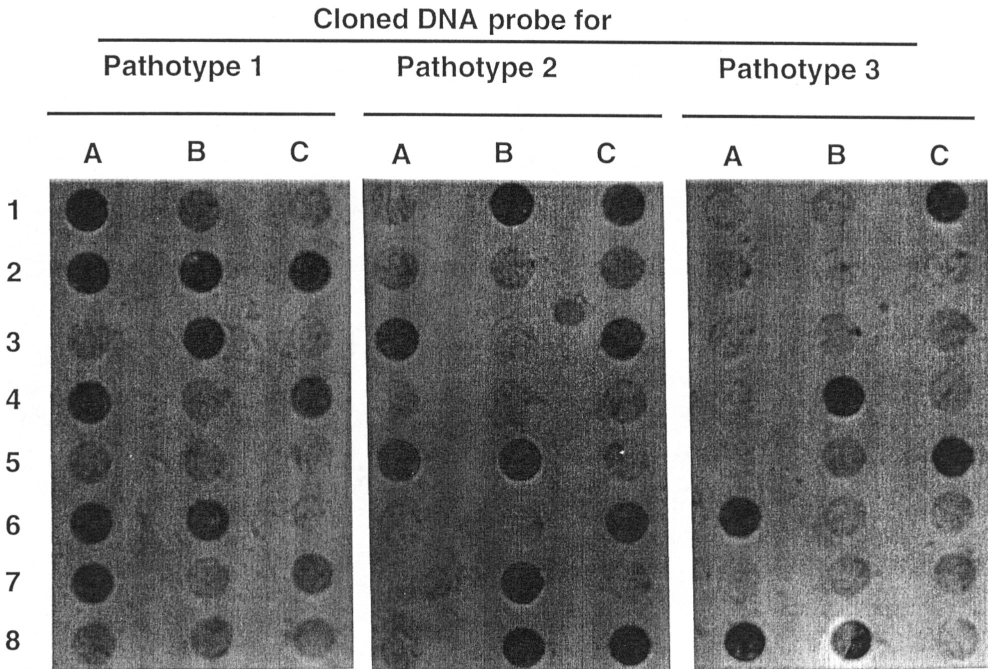


**Fig. 7.2.** A representative autoradiograph of 24 *Entomophaga grylli*-infected grasshoppers from which fungal DNA had been dot-blotted in triplicate and hybridized with a pathotype-specific cloned DNA probe. In this example there are no instances of grasshoppers co-infected with pathotypes 1 and 2. However, grasshopper A6 is co-infected with pathotypes 1 and 3, while grasshoppers B8 and C1 are co-infected with pathotypes 2 and 3.

(1992–1994) from 23% to 1.7% to 0%. Outside the 1 km radius from the release sites, no pathotype 3 infections were detected in *E. grylli*-infected grasshoppers.

Classical biocontrol entails the establishment of a fungal agent in the host population and relies on cycling of the pathogen. Obligate pathogens, such as *E. grylli*, are a good choice for classical biological control since they have restricted host ranges and show good pathogenicity toward their hosts. This strategy appears not to have applied in the *E. grylli* field trial. Infected grasshoppers from years after 1994 have not been evaluated and grasshopper populations have been relatively low up to 1998. The conidia are relatively ephemeral and are killed by high temperature, desiccation and solar radiation. However, the resting spores are reported to remain in the soil for several seasons and thus are present if grasshopper populations increase (Carruthers *et al.*, 1997). The capacity for dormancy in these fungi merits a continuing survey of pathotype 3. The decline of pathotype 3 in the host population and its purported lack of dispersal from the application area is probably good news in light of the controversy surrounding the release of exotic biocontrol agents.

As an indundative strategy the application of pathotype 3 appears not to be economically feasible since these fungi are not readily culturable and mass production must be done by injecting large numbers of grasshoppers for field release.

### *Molecular probes and the ecology of Entomophaga grylli*

It was mentioned in the introduction that the release of the lacZY-engineered strain of *P. aureofaciens* provided some of the most valuable insights into the population dynamics of rhizosphere bacteria because the fate of the clone could be monitored. The ability to distinguish *E. grylli* pathotype 3 using molecular probes not only proved to be a powerful tool for monitoring a biocontrol release but has also provided some insights into the host ranges and population dynamics of *E. grylli* (Bidochka *et al.*, 1996). For example, the degree of host specificity in various grasshopper species infected by *E. grylli* pathotypes in field conditions was shown. Cross-infections of pathotype 1 in melanopline grasshoppers were more common than pathotype 2 infection in oedipodine grasshoppers. Pathotypes 1 and 2 showed between 1.1% and 15.8% cross-infection, on a grasshopper species basis, from their preferred host. Another subfamily of North American grasshoppers, in which little information on *E. grylli* infection was known, the Gomphocerinae, were found to be most susceptible to pathotype 1 infections. Multiple infections of pathotypes 1 and 2 were very rare in grasshoppers, with only one reported case out of more than 1000 *E. grylli*-infected grasshoppers screened. This suggests that there is some mechanism operating which prevents pathotype 1 and pathotype 2 infections from co-occurring. However, multiple infections of the endemic pathotypes 1 or 2 with the exotic pathotype 3 occurred in approximately 10% (9.0% at one site and 15.5% at the other site) of the *E. grylli*-infected grasshoppers. The same stringent level of exclusion appears not to be operating between the endemic pathotypes and pathotype 3. This may indicate that some mechanism may have evolved in the endemic pathotypes during host-specific infections that excludes a competing endemic pathotype. The same principle may not apply to pathotype 3, the Australian pathotype, with the North American pathotypes because they did not co-occur and compete for hosts.

The use of cloned DNA probes also showed evidence for infection preferences by the *E. grylli* pathotypes in different life stages of grasshopper species (Bidochka *et al.*, 1996). Pathotype 2 infections occurred more frequently in early-instar *M. sanguinipes* and *Melanoplus bivitattus* than in adult grasshoppers, while pathotype 1 infections occurred more frequently in adult *C. pellucida* than in the early instars. When cross-infections (e.g. pathotype 1 in melanopline grasshoppers) or pathotype 3 infections occurred, they were found in later-instar and adult grasshoppers.

## Conclusions

Fungal biocontrol introductions require identification and monitoring of the introduced genet in the field in order to determine the effect of the pest control application and as part of a biological impact assessment. No one technique can provide conclusive identification of a fungal genet in the field against the endemic fungal population. The application of a certain technique will depend primarily on the fungus that is to be introduced into a site and information concerning the amount of variation that can be detected in the fungal species. If the fungal species is not endemic to the introduced site, techniques that can differentiate fungal species or pathotypes could be used. These include morphological markers, allozyme analysis or immunological markers. This will also depend on the ability to culture the fungus on artificial media. However, if a fungal species that is endemic to the area is to be released, more

discriminatory molecular analysis, such as RFLP, RAPD or cloned DNA probes, may be more appropriate. Techniques such as DNA arrays on genotyping chips have been developed (Chee *et al.*, 1996) and are being applied for discovering single-nucleotide polymorphisms (SNPs) in humans (Wang *et al.*, 1998). In the very near future, this technology could be accessible for screening fungal genets. There is also a vast field of knowledge devoted to modeling the fate of chemical contaminants in the ecosystem. This information could be utilized to model the fate of fungal biocontrol agents. The fallout from this type of analysis will be to provide information on the efficacy of fungal biocontrol agents and, in addition, to provide valuable information on pathogenic fungal epidemiology and ecology.

## Acknowledgements

The preparation of this article was supported by an operating grant from the National Sciences and Engineering Research Council of Canada (NSERC). Dr Fiona Hunter (Brock University), Ramya Rajagopalan and Melanie Makhija assisted in the preparation of this article.

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