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**MOLECULAR AND FUNCTIONAL ANALYSES OF INCOMPATIBILITY
GENES AT *het-6* AS GENETIC MARKERS IN POPULATIONS OF
NEUROSPORA.**

A Thesis

presented to

The Faculty of Graduate Studies

of

Carleton University

by

Nadereh Mir-Rashed

In partial fulfillment of requirements

for the degree of

Masters of Science

1998

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**The undersigned hereby recommend to
The faculty of Graduate Studies and Research
acceptance of thesis,**

**“Molecular and functional analyses of
incompatibility genes at *het-6* as
genetic markers in populations of
Neurospora.”**

Submitted by

Nadereh Mir-Rashed, B.Sc.

**In partial fulfillment of the requirements
for the degree of Master of Science**

Chair, Department of Biology

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Carleton University

"If the Lord Almighty had consulted me before embarking upon the creation, I should have recommended something simpler".

Alfonso X of Castile, 13th century (Attributed)

Abstract

MOLECULAR AND FUNCTIONAL ANALYSES OF INCOMPATIBILITY GENES AT *het-6* AS GENETIC MARKERS IN POPULATIONS OF *NEUROSPORA*.

The heterokaryon incompatibility system provides an excellent model for the molecular genetic study of non-self recognition to measure genetic variation in fungal populations. This thesis surveys genetic variation and distribution of alleles at two incompatibility genes, *het-6V* and *het-6J*, based on molecular markers in conjunction with a functional assay of *het-6* incompatibility based on partial diploid analysis. Southern blot analyses of *N. crassa* populations reveal multiple RFLPs in the regions of *het-6V* and *het-6J*. Both genes have homologous counterparts in other *Neurospora* species.

The principle result presents a pattern of genetic variation and distribution of alleles at both genes within and between worldwide population sample of *Neurospora* species. In particular, there is a high level of genetic variation, the majority of which occurs at the level of local populations.

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"A single pole cannot escape the real axis without the help of at
least one other." Trevor P.B.M. Moat

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List of Abbreviations

bp	base pair
<i>cys-3</i>	cysteine-3
<i>cot-5</i>	colonial temperature sensitive-5
DNA	Deoxyribonucleic Acid
dCTP	Deoxy-cytosine triphosphate
dNTP	Deoxy-nucleoside triphosphate
Dp	partial diploid
<i>Dp(AR18)</i>	<i>Dp(II→III)AR18</i>
EDTA	Ethylenediamine Tetraacetic Acid
FGSC	Fungal Genetic Stock Center
<i>het</i>	heterokaryon incompatibility locus
<i>HML</i>	<i>Saccharomyces cerevisiae</i> mating-type cassette (Left)
<i>HMR</i>	<i>Saccharomyces cerevisiae</i> mating-type cassette (Right)
<i>hsp-1</i>	heat shock protein-1
kbp	kilobase pairs
LGII	Linkage Group II
LGIII	Linkage Group II on left side
<i>MAT</i>	Mating-type locus
OR	Oak-Ridge
O	Other
PA	Panama
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
<i>rnr-1</i>	ribonucleotide reductase, large subunit
<i>pyr-4</i>	pyrimidine-4
SCM	Synthetic Cross Medium
TBE	Tris-borate EDTA
<i>T(AR18)</i>	<i>T(II→III)AR18</i>
<i>T(P2869)</i>	<i>T(II→IV)P2689</i>
<i>thr-2</i>	threonine-requiring mutant 2
UV	ultra violet light
un-24	unknown temperature sensitive mutation
RAPD	Randomly Amplified Polymorphic DNA

Chapter 1

General Introduction and Literature Review

General introduction

The fungi constitute a morphologically and physiologically diverse group of eukaryotes, now usually classified in a separate kingdom, Fungi. Among the attributes that distinguish the fungi are a hyphal or yeastlike form, a rigid, chitinous cell wall, often production of spores, and relatively small genome size.

The economic importance of the fungi is a reflection of their diverse, and often unique, metabolic capabilities. In nature, they are involved in symbiosis (e.g. mycorrhizae), disease, and decay and, therefore, are essential for proper ecosystem function. Fungi also play an important role in biotechnological processes, genetic and other fundamental biological research.

In the laboratory, fungi are model systems for basic research. The study of the genetics of the fungi is of value for a variety of reasons. Fungi cannot be adequately studied without knowledge of the causes of their variability; plant pathologists, plant breeders and industrial microbiologists all require an understanding of genetic variability either to prevent, or to utilize the activities of the fungi concerned. Moreover, the fungi have provided geneticists with novel genetic systems and with the opportunity of studying particular problems with greater ease or precision than is possible with other eukaryotic

characteristics of other more complex eukaryotes. Scientists who are otherwise separated by discipline, organism, geography, and objectives nevertheless share common goals in applying modern genetic techniques to fungi. The molecular biological study of fungi is just beginning and *Neurospora crassa* offers a well-developed genetic system for molecular biologists.

Due to its simple nutritional requirements (Kendrick, 1992), rapid growth rate, ease of transformation (Fincham, 1989), and its non-pathogenic nature, the Ascomycete, *Neurospora crassa*, is considered as one of the best experimental organisms for the study of eukaryotic genetics. *N. crassa* has been the subject of extensive investigation in terms of morphology, genetics and biochemistry for over 60 years. In filamentous fungi, mating type and vegetative or heterokaryon incompatibility confer self/nonself recognition in the sexual and vegetative phases, respectively. The following sections give a brief review of the *N. crassa* life cycle, the role of heterokaryon incompatibility (*het*) loci with special emphasis on the *het-6* locus.

***Neurospora crassa* life cycle**

Neurospora is a haploid, coenocytic (many nuclei per cell), filamentous Ascomycete belonging to the Class *Pyrenomycetes* and the Order *Sphaeriales*. This order includes other fungi well known to geneticists, like *Podospora*, *Sordaria*, *Gelasinospora* and *Ophiostoma*. *Neurospora* was named and described 70 years ago by Shear and Dodge

1927) soon after the sexual stage was first recognized. The genus *Neurospora* includes three heterothallic species with 8-spored asci (*N. crassa*, *N. sitophila*, and *N. intermedia*),

and one pseudohomothallic species with 4-spored asci (*N. tetrasperma*) and five true-homothallic species with 8-spored asci and without conidia (*N. terricola*, *N. africana*, *N. dodgei*, *N. galapagosensis*, and *N. lineolata*). Sexual reproduction among the heterothallic species requires the interaction of two strains that differ in mating type. In these heterothallic species, every ascus is hybrid; nuclei of different compatible strains fuse in the production of each ascus, and through meiosis, the ascospores can be recombinant for characters by which the parental strains differ. In heterothallic strains after meiosis and a single mitotic cell division, the ascus contains eight haploid ascospores, four carrying the determinants of mating type *A* and four with *a*. Sexual reproduction in homothallic species does not require fusion of different mycelia. The individuals of these species are “self fertile”. Homothallic strains are haploid and homokaryotic, that is, they contain a single nuclear type. Nuclear fusion and meiosis occur in the asci, just as in the heterothallic species, but leaving aside the possibility of mutation, the two nuclei will be identical by origin, and the resulting eight ascospores will also be identical. However, homothallic fungi can generate genetic variation through sexual reproduction.

Heterokaryosis results from the fusion of hyphae of different strains, followed by migration of nuclei from one hypha into the other, so that the hyphae come to have two kinds of nuclei. Such a hypha is a heterokaryon. By virtue of heterokaryosis, it is possible to get two different nuclei into a developing ascus. The four-spored, pseudohomothallic species *N. tetrasperma*, differs from eight-spored species in the behavior of its nuclei in the ascus (Raju, 1980). In *N. tetrasperma* all three nuclear divisions (two meiotic division and one mitosis) occur prior to ascospore delimitation, just as in the eight-spored strains, but only four ascospores are normally delimited. Each of the four ascospores encloses

two nuclei, which are usually (67% of the time) of opposite mating type. An ensuing mitotic division makes the *N. tetrasperma* ascospore four-nucleate (Raju, 1980). Thus, mycelia derived from single ascospores are often heterozygous for mating type and can produce mature perithecia without the necessity of hyphal fusion between different individuals. The pseudohomothallic condition is also referred to as secondary homothallism (Elliott, 1994).

Neurospora has been isolated in moist tropical or subtropical regions and is not frequently encountered in areas where the warm season is dry or where winters are cold with extensive freezing (Perkins and Turner, 1988). Large, orange conidiating colonies on recently burned vegetation key out almost invariably as heterothallic or pseudohomothallic *Neurospora* species. *Neurospora* may also be isolated from unburned substrates such as corncobs, sugarcane, filter mud from sugarcane refineries, or oncham (a Javanese food) (Perkins and Turner, 1988). These conidiating colonies provide a sample of the gene pool of the local population. *Neurospora* can also be isolated from soil as ascospores. This method can recover various *Neurospora* species as well as other genera whose ascospores remain dormant until exposed to heat (Perkins and Turner, 1988). Soil sampling has the advantage of contributing strains that are atypical in appearance or do not readily conidiate (Perkins and Turner, 1988).

The most widely studied species of *Neurospora* is the heterothallic *N. crassa*. This has resulted largely from the pioneering genetic work of Lindegren in the 1930s and by Beadle and Tatum who obtained the first nutritional mutants in 1941 with *N. crassa*. The life cycle of *N. crassa* includes three phases; a vegetative, or assimilative growth phase, asexual propagation and sexual reproduction phases (Figure 1.1). The vegetative phase

begins with ascospore germination following a brief period (one hour) of high temperature, for example one hour at 65°C, such as would occur during a brief exposure to fire. *N. crassa* ascospores germinate with two primary hyphae (germ tubes) growing from both ends of the spore. The primary hyphae grow by mitotic cell division and cell elongation to form a linear series of multinucleate cells. The cells of the hyphae are separated by incomplete cross-walls, or septae, which allow cytoplasm to flow between cells along the length of the hyphae carrying nuclei, mitochondria and other inclusions usually in the direction of growth (Davis and de Serres, 1962). During vegetative growth, hyphae will periodically branch or fuse with neighboring, genetically identical hyphae, to form a reticulate network. This network, comprised of many hyphae, is termed a mycelium. However, stable vegetative fusion between genetically nonidentical hyphae is usually precluded by heteroallelism at any of numerous heterokaryon incompatibility (*het*) loci or the mating-type locus (*mat*).

Asexual propagation in *N. crassa* includes mitotic cell division and dispersal by microconidia and macroconidia. Macroconidia are multinucleate propagules that arise at the tips of aerial hyphae. Macroconidia born on aerial hyphae occur in cultures on the colony surface and give *N. crassa* the characteristic orange, fluffy colony appearance. Macroconidia are used by researchers to inoculate new cultures and serve as “male” structures in fertilizing “female” trichogynes in nature and in the laboratory (Davis and de Serres, 1962). Microconidia are uninucleate asexual spores produced at the tips of short, specialized hyphae called microconidiophores and are formed by the vegetative mycelium (Davis and de Serres, 1962; Lowry *et al.*, 1967).

Figure 1-1: Generalized life cycle of *Neurospora crassa*.

Microconidia are smaller than macroconidia and have relatively poor viability. They do not become airborne and are usually produced later in the asexual phase of the fungus (Davis and de Serres, 1962).

The sexual phase begins with fertilization and concludes with ascospore differentiation. Sexual reproduction in *N. crassa* requires the participation of strains with two distinct mating types, *A* and *a*. Strains of either *A* or *a* mating type can behave as male or female partners. Self-sterile *N. crassa* of either mating type can produce male “gametes” (macro- or microconidia, hyphae or hyphal fragments) and female, reproductive structures known as protoperithecia. The sexual phase is triggered by low nutrient condition, particularly when either carbon or nitrogen becomes limiting for vegetative growth. This environmental stress appears to initiate a series of biochemical changes, which leads to the formation of protoperithecia. Gametic fusion is accomplished when a male gamete of one mating-type comes into contact with a trichogyne, a specialized receptive hypha arising from the protoperithecium, of the other mating-type. Following fusion of the male gamete and trichogyne, the “male” nucleus moves down the trichogyne. Within the protoperithecium, *A* and *a* nuclei divide 8-10 times before fusing and undergoing meiosis (Glass and Lorimer, 1991). The final, conjugate division of *A* and *a* nuclei before ascus formation occurs in a hook-shaped cell called the crozier. These croziers give rise to asci in an intricate manner (Raju, 1980). Fusion of the *A* and *a* nuclei results in a brief diploid phase and meiosis. Meiosis is followed by a single mitotic division, to give rise to eight haploid ascospores, four of each mating type arranged as a linear tetrad in an ascus (Glass and Lorimer, 1991). The asci are sack-like structures contained within the perithecium. In *N. crassa* and related Pyrenomycetes mature

perithecia are characterized as being superficial, flask-shaped bodies with a black pigmentation (Glass and Lorimer, 1991). A mature perithecium develops a beak terminating in a pore, the ostiole, through which asci protrude in turn to forcefully eject the ascospores. Upon germination, each ascospore can give rise to a haploid mycelium.

Genetics of *N. crassa*

The original auxotrophic mutants of *N. crassa* were obtained by Beadle and Tatum (1945) and led to the famous “one gene, one enzyme” hypothesis. Through genetic mapping of an expanded set of auxotrophic markers, six linkage groups were identified by 1949 and the seventh was soon added (Reisner, 1953). The haploid genome of *N. crassa* consists of seven chromosomes with a total genetic map length of roughly 1000 map units (Selker, 1990) and about 38,900 kbp of DNA (Orbach, 1993). The genome consists of 2% foldback, 8% repetitive (90% of which is ribosomal DNA), and 90% single copy sequences (Krumlauf and Marzluf, 1980). The genetic map of *N. crassa* was the first fungal genetic map to have all linkage groups including over 700 loci and breakpoints of more than 300 rearrangements (Perkins, 1992). Perkins (1982), Fincham and Day (1971), Davis and de Serres (1970) have published useful linkage maps of *N. crassa*.

Gene order can be resolved most easily through analysis of duplication coverage (Perkins *et al.*, 1986) with the breakpoint of a chromosome rearrangement as a reference point. If a rearrangement with appropriate breakpoints is available, determining the order of closely linked genes by duplication coverage (partial diploids) can be straightforward. In fact, a number of heterokaryon incompatibility genes in *N. crassa*, including *het-6*, were discovered and mapped by chromosome duplications (Mylyk, 1975). The incompatibility

of the genes in heterokaryons is paralleled by their incompatibility in duplications.

Duplications homozygous for the genes are normal in growth and morphology.

Duplications heterozygous for these loci are inhibited in initial growth and produce brown pigment on an appropriate medium, and later exhibit “escape” from their inhibition (Mylyk, 1975). Escape occurs when one or the other of the duplicated regions is deleted (Smith *et al.*, 1996). In *Neurospora*, partial diploid progeny are synthesized from crosses between a normal sequence strain and a strain with an insertional or a terminal translocation.

There are three main types of chromosome rearrangements; insertional translocations, in which a terminal or interstitial portion of one chromosome is inserted into another chromosome [e.g. $T(III \rightarrow IIIIR)AR18$], reciprocal translocations, in which the terminal portion of two chromosomes are exchanged [e.g. $T(IR;IIR)STL76$], and inversions, in which a portion of a chromosome is inverted with respect to the wild-type arrangement [e.g. $In(IL \rightarrow IR)NM176$]. Mapping by duplication coverage has been used extensively in *N. crassa* for locating new mutants and for resolving ambiguities in regions where gene order was uncertain. This is reflected in recent *Neurospora* linkage maps where numerous gene loci are shown relative to the breakpoints of duplication generating rearrangements (Perkins *et al.*, 1982; Perkins, 1986). Conventionally, ordered tetrads have been employed in *Neurospora* for assigning gene loci to left or right chromosome arms (Barratt *et al.*, 1954).

Genetic analysis with *Neurospora* has an advantage over the classic materials of genetics in that the four products of each meiosis remain together as a tetrad of ascospores and can be analyzed directly. Further, in some instances particular exchange

and the distribution of chromatids can be inferred from the order of genetic markers among the spores in the ascus. This is possible when the spindles in both meiotic divisions and the postmeiotic mitosis are so oriented that the nuclei, and thus the spores, assume a specific and predictable order (Bos, 1996). Such groups of nuclei or spores are called “ordered tetrads” (Elliott, 1994). In *N. crassa*, the segregation pattern of mutants that differ from the wild type in spore characters (e.g. color, size) can be determined directly by viewing the spores in the asci. Mapping by ordered tetrad analysis becomes more difficult as the gene-centromere distance decreases (Perkins, 1986). Finally, generating mutations in the laboratory through UV treatment, X-Ray exposure or neutron bombardment, with subsequent isolation of single strains on complete medium identified many of the loci represented on the *N. crassa* genetic maps (Beadle and Tatum, 1945).

Heterokaryon Incompatibility (*het*) in *N. crassa*

Heterokaryon incompatibility results in the inability of two genomes to coexist in a cell (Buss, 1982; Grosberg, 1988). Heterokaryon incompatibility may be considered as a form of incompatibility, or non-self recognition, a phenomenon that is widespread in biological systems (Buss, 1982; Grosberg, 1988) and probably results from various physiological mechanisms. In general, incompatibility can be considered at four levels; fusion failure, transplant failure, fertilization failure, and offspring lethality.

Fusion and transplant failure are expressed in higher eukaryotes by incompatibility reactions following tissue contact between genetically different conspecifics. Esser (1959) distinguished two basic types of genetic control of incompatibility in fungi, homogenic and heterogenic. Homogenic incompatibility, in which sexual interaction is prevented by the

same alleles at an incompatibility locus, may be considered as a type of fertilization failure. Sexual mating in *N. crassa* is controlled by a homogenic incompatibility system; mating occurs only between strains that differ at *mat*. Heterogenic incompatibility systems prevent hyphal interaction or fusion when different alleles occur at incompatibility loci. Heterogenic incompatibility has been studied in a number of fungi, especially *Neurospora crassa* (Garnjobst and Wilson, 1956), *Podospora anserina* (Bégueret *et al.*, 1994), *Aspergillus nidulans* (Grindle, 1963) and *Cryphonectria parasitica* (Anagnostakis, 1977; Milgroom and Lipari, 1993). At least two forms of heterogenic incompatibility are evident among fungi, heterokaryon and vegetative incompatibility, and these differ at least in the ways they are recognized, and perhaps in their genetic and biochemical mechanisms as well. Heterokaryon incompatibility is evident when vigorous, stable heterokaryons do not form because of differences at *het* loci. Vegetative incompatibility occurs when different fungal strains are grown on the same petri dish and a “barrage” line is formed at the zone of contact between two strains. This “zone line”, as it is sometimes called, may be evident as a line of pigmentation or as a clear zone.

In this thesis, the genetic markers used are of *het* genes and so the genetic and biological background of heterokaryon incompatibility will be described below. In *Neurospora* heterokaryons are rarely isolated from nature (Caten and Jinks, 1966), and the formation of heterokaryons between different wild strains of a species is normally restricted. There have been ten *het* loci (the loci governing heterokaryon incompatibility) identified in *N. crassa* (Mylyk, 1975). The incompatibility due to these genes occurs only during the vegetative phase of the life cycle and does not reduce fertility. In *N. crassa*, the *A* and *a* mating-type genes also act as heterokaryon incompatibility genes during the

vegetative phase of the life cycle (Glass and Lorimer, 1991). Heterokaryon incompatibility occurs when strains differ at any one of the ten *het* loci or at *mat*. In most cases, only two alternative alleles have been identified at *het* loci, however there is genetic evidence for multiple alleles at *het-C* and *het-8* (Howlett *et al.*, 1993).

Research on heterokaryon incompatibility in *N. crassa* has focused on the number, location, and phenomenology of the different loci and alleles. Three general procedures are used for this: 1) heterokaryon tests, where heterokaryons are examined for prototrophic growth (Garnjobst, 1953); 2) through examination of partially disomic strains that are heterozygous at one or more *het* loci (Perkins, 1975; Mylyk, 1975); 3) microscopic examination of incompatibility reactions (Garnjobst and Wilson, 1956; Jacobson *et al.*, 1997).

The heterokaryon test involves co-inoculation of two strains that have complementary auxotrophic markers on to a minimal medium that will not permit growth of either strain alone. If paired mycelia differ at any of the ten *het* loci or at *mat*, a lethal cytoplasmic incompatibility reaction follows hyphal anastomosis (hyphal fusion), effectively preventing the formation of a heterokaryon (Garnjobst and Wilson, 1956). Garnjobst and Wilson (1956) first showed those allelic pairs at two specific loci, *het-C* and *het-D*, will prevent heterokaryon formation. For example, they found that strains carrying *C* and *D* alleles form heterokaryons with each other but not with strains of *Cd*, *cD* or *cd* genotypes.

Heterokaryon incompatibility can also be demonstrated in a single nucleus in which at least one *het* locus has been duplicated. Duplications of opposite alleles of a *het* locus are accomplished by creating partial diploids (Mylyk, 1975). In *Neurospora*, this is done

by crossing a normal sequence strain to a strain with a nonreciprocal chromosomal translocation, which includes a *het* gene. One third of viable progeny inherit the chromosome with the endogenous *het* locus and the chromosome with the translocated *het* locus, hence two copies of a *het* gene. If the *het* alleles are different, these partial diploid progeny have an incompatible duplication within the same nucleus. The phenotype of these duplication progeny is termed self-incompatible or “dark agar” and is characterized by slow growth and the production of a diagnostic dark pigment on a tyrosine/ phenylalanine agar (Perkins, 1975). The loci *het-5*, *het-6*, *het-7*, *het-8*, *het-9* and *het-10* were originally identified by this method (Mylyk, 1975).

After fusion of incompatible cells, light microscopy reveals the fusion cell is sealed off from the rest of the hyphae. The fusion cell soon becomes vacuolated and then undergoes plasmolysis and death. Heterokaryon incompatibility does not, therefore, appear to occur through prevention of fusion. In fusion involving compatible strains, protoplasmic and nuclear exchange occurs across the fusion cell and results in the formation of a stable heterokaryon. Wilson (1961) showed that transplantation by microinjection of nuclei alone could bring about this reaction. Jacobson *et al* (1997) investigated microscopically the heterokaryon incompatibility reaction in partial diploids heterozygous at *het-6* or *het-c* in *N. crassa*. Electron microscopy showed organelle degradation and plasmolysis. Inhibited duplications were employed because of the difficulty in direct examination of cell fusion. There were no noticeable differences in the ultrastructure of cell undergoing incompatibility reactions due to allelic differences at *het-6* and *het-c*. This is surprising because different alleles at *het-6* result in a much stronger incompatibility reaction than incompatibility at *het-c*. This observation suggests that

different incompatibility genes may trigger the same pathway to cell death (Jacobson *et al.*, 1997).

The value of heterokaryon incompatibility in fungi

Heterokaryon incompatibility plays an important role in fungal biology. In Basidiomycetes and some other fungi in which specialized sexual organs do not differentiate, heterokaryosis is the first stage of the sexual phase and is controlled by mating-type systems. In imperfect fungi, with no known sexual phase, heterokaryosis also provides a means to initiate the parasexual cycle and generate genetic diversity through mitotic recombination. It has been proposed that heterokaryon incompatibility protects homokaryons from infection by viruses or other disease elements (Caten, 1972; Day, 1970). Caten (1972) showed that vegetative death in *Aspergillus*, a progressive decline in growth rate and vigor determined by a transmissible cytoplasmic factor, is readily transmitted among heterokaryon compatible strains, but transmitted at a lower frequency between partially compatible, and not at all between incompatible strains.

Anagnostakis (1977) found evidence for at least 28 different vegetative compatibility groups in the chestnut blight pathogen, *Cryphonectria parasitica*. During this century, epidemics of chestnut blight have devastated chestnut trees in both North America and Europe. Vegetative incompatibility in *C. parasitica* may well prove to be a major obstacle to the use of hypovirulence, known to be determined by a double stranded RNA virus, as a biological control of chestnut blight. Biocontrol of chestnut blight by spreading hypovirulent strains has been more effective in Europe than in North America. Studies on the number of heterokaryon incompatibility groups of *C. parasitica* indicate

that there are more heterokaryon incompatibility groups in North America than in Europe (Anagnostakis *et al.*, 1986). The viral RNA is, as far as is known, transmitted only by hyphal anastomosis. Since vegetative incompatibility makes the products of anastomosis inviable, *het* differences may severely restrict the spread of viral elements in nature. Since viruses and other disease elements are comparatively common in the fungi, it would not be surprising to find that barriers to their spread, such as *het* genes, had been selected in nature.

Population biology of fungi

Being one of the quantitative branches of biology, population genetics makes extensive use of understanding populations of organisms. While experimental population genetics has been invigorated by the application of molecular methods, theoretical population genetics has also undergone resurgence because the molecular data make it possible to test many theoretical predictions. At the descriptive level, population genetics involves studies of the amount of genetic variation present in populations, and of the spatial and temporal distribution of these variations. In this view, filamentous fungi offer excellent opportunities for studying the effects of the genetic system on genetic population structure. The study of population biology of fungi seeks to understand and predict the effects on populations of such genetic phenomena as segregation, recombination, transposition and mutation. Such studies take into account ecological and evolutionary factors such as population size, patterns of mating, geographical distribution of individuals, migration, and natural selection.

The fungi have been largely ignored in the literature of population and evolutionary genetics, mostly because essential information on fungal populations has been lacking. Population genetic research of fungi started with research on *Aspergillus* in Europe around 1970 (Jinks *et al.*, 1966; Caten, 1971; Croft and Jinks, 1977) and in North America on *Neurospora* (Perkins *et al.*, 1976; Perkins and Turner, 1988). With the development of molecular genetic markers, interest in the population genetics of plant pathogenic fungi has grown substantially in the past several years. The most intensively studied fungal populations include plant pathogenic fungi such as *Fusarium proliferatum*, *Armillaria* spp., *Heterobasidion annosum* and *Cryphonectria parasitica*. Plant pathogens provide opportunities to study many basic processes in population and evolutionary biology. One aim to the study of plant pathogenic fungi is to design strategies for disease control.

Genetic analysis of fungal populations requires the identification of genetic markers, which are unambiguous and informative. Such genetic markers provide information that is useful to survey the relationship between genotype and phenotype, and about the genetic structure of fungal populations (Kolmer, 1991). Many molecular genetic markers have been used for this including isozymes, Restriction Fragment Length Polymorphisms (RFLPs), DNA fingerprints and Randomly Amplified Polymorphic DNAs (RAPDs). These molecular markers allow measurement of the amount and distribution of genetic variation in populations of fungi. For example, in *Mycosphaerella graminicola*, variation at individual RFLP loci and DNA fingerprints were used to show that thousands of different genotypes may occur in individual fields of wheat (Boeger and McDonald, 1991; McDonald and Martinez, 1991). A similar pattern for the amount and distribution

of genetic variation was found using RFLPs and DNA fingerprints in a population of *Stagonospora nodorum*, which causes wheat leaf and glume blotch (McDonald and McDermott, 1993).

Purpose and objectives

It was hypothesized that genetic variation in *Neurospora crassa* can be investigated by the study of *het* genes as genetic markers. Since *het* genes are a means by which a fungus recognizes self from non-self, analysis of genetic markers associated with these genes should provide information relevant to fungal individuals and populations.

The objectives for this study are as follows:

1. To develop molecular markers of *het-6J* and *het-6V* for use in study of *N. crassa* populations. *het-6J* and *het-6V* are two genes located close together, at what has been referred to as the “*het-6*” locus.
2. To examine the relationship between incompatibility function at *het-6* based on partial diploid analysis, and molecular markers at *het-6J* and *het-6V*.
3. To study the number of alleles at *het-6J* and *het-6V* and whether combination of alleles at these two genes provides additional functional varieties of *het-6*.

The objectives of each chapter are as follows:

Chapter 2- To investigate genetic variation and distribution of alleles at *het-6J* and *het-6V* based on molecular markers in conjunction with a functional assay of *het-6* incompatibility based on partial diploid analysis in a population sample of *N. crassa* from Louisiana.

Chapter 3- To determine the genetic distribution and variation of alleles at *het-6J* and *het-6V* by RFLP analysis in *N. crassa* isolates from 13 sites through the tropical Americas, 2 sites in Africa and 7 sites in tropical Asia. In addition, the distribution of restriction fragments that hybridize to *het-6J* and *het-6V* probes in other *Neurospora* species, and closely related, and distantly related fungi was examined.

It is hoped that this study will encourage wider studies in the genetics of fungal populations and will contribute to an increased appreciation of the function of *het* genes and their potential use as molecular markers of the non-self recognition system.

Chapter 2

Molecular and functional analyses of incompatibility genes at *het-6* as genetic markers in a population of *Neurospora crassa*.

Introduction

Neurospora crassa is genetically and biochemically one of the best characterized fungi. However, relatively few studies examine natural population of *Neurospora crassa*. Additional study of *N. crassa* populations would contribute significantly to the understanding of evolutionary biology in several ways. From a global point of view, the study of haploid eukaryotes provides comparisons with the diploid eukaryotes with which population geneticists have been largely concerned. Chromosome organization, meiosis, and genetic recombination in *N. crassa* are basically similar to that of plants and animals. *Neurospora crassa* is a non-pathogenic fungus, naturally occurring in the tropical and subtropical environment with a variety of features similar to pathogenic fungi. As far as the relationship of the fungi to human affairs, the study of *Neurospora crassa* populations may bring new insights into population biology of pathogenic fungi. How do pathogenic and non-pathogenic fungi differ? In identifying those differences, we may gain insight into the pathogenic life style. While the available genetic background information and abundant resources provide useful tools for design and study of *N. crassa* populations, it is expected that further understanding of naturally occurring genetic diversity in *N. crassa* will provide useful material and insight for molecular genetic and biochemical studies. It is

with this in mind that we have begun to examine populations of *N. crassa* using molecular and functional marker of genes involved in non-self recognition.

Non-self recognition occurs in animals, protists, plants, bacteria and fungi (Buss, 1982; Grosberg, 1988; Clewell, 1993; Mahall and Callaway, 1996). As in many other organisms, non-self recognition in filamentous fungi occurs during both the asexual and sexual phases of the life cycle. The heterokaryon incompatibility system in *Neurospora crassa* is a genetically well characterized nonself recognition system that is involved in limiting heterokaryosis (Perkins, 1988). An allelic difference at any one of at least ten heterokaryon incompatibility (*het*) loci or at the mating-type locus (*mat*) in *N. crassa* prevents the stable growth of heterokaryotic hyphae during the vegetative phase of the life cycle. Interestingly, *het* differences do not prevent fertilization or the limited heterokaryosis that occurs during the sexual cycle and a difference at *mat* is essential for entrance into the sexual cycle.

In this study, we examine characteristics of the *het-6* locus using a functional assay and molecular genetic markers with forty *N. crassa* strains sampled from a natural population from a Louisiana sugarcane field. Mylyk (1975) discovered *het-6* on the left arm of linkage group II, (LG II, Figure 2-1) through partial diploid analysis. This involves examining for self-incompatible progeny from a cross between a wild type, normal-chromosome strain and a translocation strain. If the parental strains differ at a *het* gene within the translocated segment, one third of viable progeny will be self-incompatible, characterized by slow growth and abnormal morphology. Two genes, *het-6V* and *het-6J* identified at *het-6* have incompatibility activity in transformation assays (Smith *et al.*, unpublished). *het-6J* and *het-6V* occur about 14 kbp apart within a 35 kbp region that

identifies the location of heterokaryon incompatibility function at *het-6* (Smith *et al.*, 1996; Smith *et al.*, unpublished). Both genes possess at least two possible allelic variants, Oak-Ridge (OR) and Panama (PA), which are incompatible when they occur together in the same cell. We hypothesize that genetic variation in *Neurospora crassa* can be investigated by the study of *het-6* as representatives of heterokaryon incompatibility genes. Our objectives in this study were threefold. First, we wanted to develop molecular markers that discriminate alleles at *het-6J* and *het-6V*. Second, we wished to examine the relationship between incompatibility function, based on partial diploid analysis, and these molecular markers. Third, we were interested to examine the number of alleles at *het-6* and to determine whether combinations of alleles at *het-6J* and *het-6V* provide additional functional variation at *het-6* in general.

Figure 2-1: Selected genetic markers on left arm of linkage group II in *Neurospora crassa* (Perkins *et al.*, 1982 and M. L. Smith personal communication). Translocated segments in $T(II \rightarrow III)ARI8$ and $T(II \rightarrow VI)P2869$ are indicated below the map. Map is drawn to approximate scale where genetic distance between *pyr-4* and *het-C* is about 1 map unit.

----- *T(II→III)AR18*

----- *T(II→VI)P2869*

Methods and Materials

Strains, Media and Culture Conditions

A population sample of forty *N. crassa* strains was collected as masses of conidia from separate distinct colonies on burnt sugarcane stubble in a five hectare field near Franklin, Louisiana (Marcenko-Kuehn *et al.*, 1994). Fifty-four strains were cultured, purified, and identified to species using standard methods (Perkins and Turner, 1988). All these strains (Table 2-1) have been deposited in the Perkins collections (numbers P4448-P4501) which is now being curated at the Fungal Genetics Stock Center (FGSC, Department of Microbiology, University of Kansas Medical School, Kansas City, Kansas). Other strains used in this study include the *het-6*^{PA} and *het-6*^{OR} standard strains, RLM58-18 (Smith *et al.*, 1996) and 74-OR28-IVA (FGSC 2489), respectively. The translocation strains *T (II→VI)P2869* (FGSC 1828A and 1829a) and *T (II→III)ARI8* (FGSC 2643A and 2644a) were used in crosses with all Louisiana strains. *N. crassa* strains were grown in either solid or liquid Vogel's medium and crosses were performed on Synthetic Cross Medium (SCM) using standard methods (Davis and de Serres, 1970; Perkins 1986). Partial diploid analyses presented in this chapter were done by Dr. D. Jacobson, Department of Biology, Stanford University. For partial diploid analyses, a wild strain and the translocated strain of opposite mating type were inoculated to a 13 × 100 mm tube containing 2.5 ml SCM. Crosses were incubated at 25°C for at least 4 weeks to allow ascospores to shoot and mature. For each cross, 100 ascospores were placed in a equally spaced grid pattern on a single 9 cm diameter petri dish containing minimal medium supplemented with sorbose (1%) to restrict colony size and tyrosine (0.5 g/L) and

phenylalanine (0.2 g/L) to encourage inhibited progeny to produce brown pigment. The entire plate was heat shocked for 40–45 min at 60°C and subsequently incubated for 2 days at 34°C. Ascospore progeny were then examined under a dissecting microscope (up to $\times 60$) to assess germination, growth, and colony morphology. Wild type versus inhibited morphology was confirmed after an additional 1–4 days incubation at room temperature (22°–24°C). Inhibition colonies only developed brown pigment after 3–4 days growth on this medium.

DNA Isolation

Mycelia grown in liquid medium were harvested by vacuum filtration, washed two times with 0.9% NaCl, dried under vacuum overnight, and pulverized to a fine powder using a glass rod. *Neurospora* DNA extractions of powdered mycelium were by the method of Oakley *et al.* (1987). Relative concentrations of genomic DNAs were quantified after agarose gel electrophoresis and staining with ethidium bromide by visual approximation over a long-wavelength ultraviolet light source.

RFLP Analysis

Genomic DNA extracts were digested separately with *EcoRI* and *HindIII* (Gibco/BRL, Burlington, ON), by using the appropriate reaction conditions and incubation times of 1 to 2 hours. Fractionation of the restriction enzyme-digested DNAs was carried out by electrophoresis in 0.7% agarose with a 1 $\times$ Tris-acetate/EDTA buffer at 1 V/cm for 16 to 17 hours. Agarose gels were stained with ethidium bromide, photographed over UV light prior to capillary transfer of digested DNAs to nylon

membranes (Hybond-N⁺, Amersham, England) by the method of Southern (1975). DNA probes were labeled with α -[³²P] dCTP by nick translation (Bethesda Research Laboratories, Gaithersbourg, MD) or by the random primer method using T7 Quick Prime (Pharmacia, Baie d'Urfe, P.Q.). Prehybridization and hybridization conditions were done according to the membrane manufacture's recommended procedure. Autoradiography was performed with Kodak Bio-Max-1 film at -80°C for 1 to 4 days. Fragment mobility was measured as the migration distance from the bottom of the loading well in the agarose gel to the center of the hybridization signal. Molecular size of each fragment was estimated by comparison with the molecular size standard of *Hind*III- digested phage lambda DNA (Boehringer Mannheim).

Polymerase Chain Reaction (PCR) amplification

Fifteen oligonucleotides were synthesized with an Applied Biosystems 390 RCR-mate DNA synthesizer (Molecular Genetics Laboratories at Carleton University, Biology Department) based on the DNA sequence of Oak-Ridge alleles at *het-6V* or *het-6J*. From these, 2 sets of primers were selected on the basis of efficiency in the characterization of samples (Figure 2-2). For *het-6V*, primers 6VP3 (5'-CGGTAACCTGTTTCAGCT-3') and 6VP5 (5'-CCCGCTAAGCCAAGGAGTCC-3') were used. For *het-6J*, primers 6JP6 (5'-GTGCGGGCTTAACCGCTG-3') and 6JP11 (5'-CTCCGGATGAGGTTGCCG-3') were used.

Figure 2-2: Inserts of plasmids p8AS-1 (contains all of *het-6V*) and pECO16M (contains all of *het-6J*) showing selected restriction enzyme recognition sites. The annealing position of primers 6VP3 and 6VP5 are given below the p8AS-1 insert and *Mbo*I sites within the primer pair are indicated. Primers 6JP11 and 6JP6 are shown below pECO16M and the position of *Msp*I sites within the primer pair are given.

p8AS-1 insert (*het-6V*)

1 kbp

pECO16M insert (*het-6J*)

For *het-6V*, PCR was carried out in a final reaction volume of 50 μ l and included about 100 ng *N. crassa* genomic DNA, 200 μ M of each dNTP, 10 μ M each of primers 6VP3 and 6VP5, and 1.25 units of *Taq* DNA polymerase (Gibco BRL, Burlington, ON). PCR reactions were done in a Barnstead/ Thermolyn Thermocycler (Ducuque, IA) programmed for 30 cycles of; 15s at 95°C (denaturation), 30s at 48°C (annealing) and 2 min /1 kbp DNA at 72°C (extension). Amplification products were analyzed by agarose gel electrophoresis with and without digestion with *Mbo*I restriction enzyme.

For *het-6J* PCR reaction volume was 50 μ l and the amplification conditions were, 300 nM of each primer, 200 μ M of each dNTP, 1 $\times$ reaction buffer with MgCl₂ and 2.27 units Expand DNA polymerase mix (Boehringer Mannheim). Between 60 ng and 200 ng of *N. crassa* genomic DNA was used for reactions. The same thermocycler parameters were used for *het-6J* as described for *het-6V* except the annealing temperature was changed from 48°C to 58°C. About 2 μ g of each *het-6J* PCR product was digested with 20 units of *Msp*I restriction endonuclease and subjected to electrophoresis in 0.75 $\times$ 20 $\times$ 20-cm, 10% non-denaturing polyacrylamide gels containing 10% glycerol, 0.5% agarose and 1 $\times$ TBE (0.095 M Tris-borate, 0.002 M EDTA, pH 8.0). Electrophoresis condition were 200V, 15 mA for 10 minutes, then 180V, 14 mA for 1 hour followed by 140V, 10 mA for 2 days all at 4°C while recirculating the buffer. After electrophoresis the gels were stained with 10-mg/ml of ethidium bromide in 1 $\times$ TBE buffer for 45 to 60 minutes and then destained in distilled H₂O for 45 minutes. DNA bands were observed and photographed over ultraviolet light as described previously.

Results

Partial Diploids Analysis

Partial diploids were obtained by crossing the 40 Louisiana wild type strains of *N. crassa* with translocation strains of *T(II→VI)P2869* [hereafter referred to as *T(P2869)*] and *T(II→III)AR18* [hereafter referred to as *T(AR18)*]. The translocation strains *T(P2869)* and *T(AR18)* are known to produce partial diploids for the *het-6* region (Mylyk, 1975; Perkins, 1975). The translocated segment in *T(P2869)* has a right breakpoint that is within 5.6 kbp of the right breakpoint of *T(AR18)* (Smith and Glass, 1996) but extends distally beyond the left breakpoint of *T(AR18)* toward the left telomere region of LGII (Perkins *et al.*, 1982) (Figure 2-1). Both *T(P2869)* and *T(AR18)* are *het-6^{OR}* (Mylyk, 1975). From the wild-type × translocation strain crosses, it is expected that one third of viable progeny will contain the translocated segment as duplication and these progenies with duplications are termed “partial diploids”. If the normal sequence (wild-type strain) and the translocation strain contain different alleles at *het-6*, the partial diploid will be heterozygous for *het-6* and produce an inhibited colony. Such colonies are termed “autoincompatible” or “self-incompatible”. In the first round of partial diploids analyses, progeny from wild-type × *T(P2869)* were examined for self-incompatibility. Twenty Louisiana wild-type strains were observed to produce a substantial proportion of inhibited progeny. It was tentatively concluded from this, that the *het-6* alleles in these wild-type strains were functionally different from the *het-6^{OR}* allele in *T(P2869)*.

However, since *T(P2869)* duplicates a large segment of LGIII, it is possible that inhibited progeny may be caused by heterozygosity not only at *het-6*, but also at any as yet undescribed *het* loci centromere distal to *het-6*.

To confirm that an allelic difference at *het-6* was responsible for the observed inhibited progeny, the 20 non-Oak Ridge isolates were crossed with the insertional translocation *T(AR18)* which duplicates a much smaller LGIII segment centered around *het-6* (Smith and Glass, 1996). Crossing and progeny analysis were as described above. All duplicated *AR18* (*DpAR18*) have an inhibited, colonial phenotype even when homozygous at *het-6*. However, *DpAR18* that are heterozygous at *het-6*, are more inhibited and produce brown pigment as they age. This difference could not be identified on the sorbose plates. Therefore, each inhibited colony was transferred from the sorbose plate to a tube of complete medium and incubated at 25°C. Classifying the inhibited phenotype required *DpAR18* cultures to be observed daily, for up to 7 days, and compared to *DpAR18* cultures of both homozygous and heterozygous *het-6* genotypes obtained from crosses with *het-6*^{OR} and *het-6*^{PA} standard strains. For all 20 strains, inhibited duplication progeny were produced in crosses to both *T(P2869)* and *T(AR18)*, confirming that no loci centromere distal to the *T(AR18)* breakpoint were responsible for the inhibited phenotype. From this, it was inferred that 20 strains carried a non-Oak Ridge allele and the remaining 20 strains carried the OR allele at *het-6* (Table 2-1). In Table 2-1, the non-OR allele is designated as PA, since it is indistinguishable from the Panama allele in the *het-6*^{PA} standard strain based on the partial diploid assay.

Table 2-1: Louisiana population sample and Oak-Ridge and Panama standard strains used in this study and associated partial diploids phenotype, RFLP, PCR-RFLP markers.

Strains	<i>het-6</i>		RFLP		PCR-RFLP	
	<i>& mat</i> ¹	function ²	<i>het-6V</i>	<i>het-6J</i>	<i>het-6V</i>	<i>het-6J</i>
<u>Louisiana strains</u>						
4448 <i>a</i>	PA ³	nt ⁴	nt	PA	PA	nt
4449 <i>A</i>	PA	A	L	PA	PA	PA
4450 <i>a</i>	OR	F	N	OR	OR	OR
4451 <i>a</i>	OR	G	N	OR	OR	OR
4452 <i>a</i>	PA	A	L	PA	PA	PA
4453 <i>A</i>	OR	G	N	OR	OR	OR
4454 <i>A</i>	OR	H	N	nt	nt	OR
4455 <i>a</i>	OR	F	N	OR	OR	OR
4456 <i>a</i>	OR	H	N	OR	OR	OR
4457 <i>a</i>	OR	F	L	OR	OR	OR
4459 <i>a</i>	OR	G	N	OR	OR	OR
4463 <i>A</i>	OR	nt	M	OR	OR	OR
4464 <i>A</i>	OR	I	M	OR	OR	OR
4465 <i>a</i>	OR	E	M	OR	OR	OR
4467 <i>A</i>	OR	F	N	OR	OR	OR
4468 <i>A</i>	PA	A	L	PA	PA	PA
4469 <i>a</i>	PA	A	L	PA&OR	PA	PA
4470 <i>a</i>	OR	E	nt	OR	OR	OR
4471 <i>a</i>	PA	A	N	PA	PA	O
4472 <i>a</i>	PA	C	nt	PA	PA	O
4476 <i>a</i>	PA	A	nt	nt	nt	PA
4479 <i>a</i>	PA	A	L	O	O	O
4480 <i>a</i>	OR	G	N	OR	OR	OR
4481 <i>a</i>	PA	nt	N	PA&OR	PA	PA
4483 <i>a</i>	OR	G	N	OR	OR	OR
4484 <i>A</i>	OR	nt	nt	OR	OR	OR
4485 <i>A</i>	PA	B	L	PA	PA	PA
4486 <i>A</i>	PA	A	nt	PA	PA	PA
4487 <i>A</i>	OR	nt	nt	OR	OR	OR
4489 <i>a</i>	PA	A	L	PA	PA	PA
4490 <i>A</i>	PA	B	L	PA	PA	PA
4491 <i>a</i>	OR	G	N	OR	OR	OR
4493 <i>a</i>	PA	nt	nt	PA	PA	PA
4494 <i>A</i>	PA	B	U	PA&OR	PA	PA
4496 <i>a</i>	OR	E	M	OR	OR	OR
4497 <i>a</i>	PA	nt	nt	PA	PA	O
4498 <i>a</i>	PA	nt	nt	PA	PA	PA

[Continued on next page...]

[... Table 2-1 continued]

4499 <i>a</i>	OR	G	N	OR	OR
4500 <i>a</i>	PA	nt	nt	PA	PA
4501 <i>a</i>	PA	nt	nt	PA	PA

Standard strains

RLM58-18 <i>a</i>	PA	A	L	PA	PA
74-OR23-IV <i>A</i>	OR	F	L	OR	OR

¹*mat*= mating-type, ²based on partial analysis, ³strains that are not OR at *het-6* are designated here as PA, ⁴nt=not tested.

RFLPs at *het-6V*

Two genes, *het-6V* and *het-6J*, at the *het-6* locus are known to have incompatibility activity in transformation assays (Smith *et al.*, unpublished). These genes are separated by about 14 kbp with *het-6J* being centromere distal to *het-6V*. To examine RFLPs around *het-6V*, the *Hind*III restriction enzyme was selected for which no recognition sites are located within *het-6V*^{OR}. Genomic DNA of 40 *N. crassa* samples from Louisiana and *het-6V*^{OR} and *het-6V*^{PA} standard strains, 74-OR23-IVA (Oak-Ridge) and RLM58-18 (Panama), respectively, were digested with *Hind*III. The digestion products were subjected to electrophoresis, Southern blotted and then hybridized with radiolabeled p8AS-1, which contains the entire *het-6V*^{OR} open reading frame (Figure 2-2). The RFLP profiles at *het-6V* for 30 of the Louisiana *N. crassa* strains were determined and are summarized in Table 2-1. RFLP patterns at *het-6V* for each strain could be placed into eight classes, A, B, C, E, F, G, H or I, based on the restriction fragment patterns. Representatives of each of these classes are shown in Figure 2-3. The numbers of fragments and fragment sizes that define these classes are given in Table 2-2. Nine strains were classed as A, three as B, one as C, three as E, four as F, seven as G, two as H and one as I. The Oak-Ridge and Panama standard strains fall into classes F and A, respectively. These classes can be correlated to the *het-6* Panama and Oak-Ridge alleles as determined by partial diploid analyses; strains in classes A, B or C are all *het-6*^{PA}, strains classed as E, F, G, H or I are all *het-6*^{OR}. All but two *het-6*^{OR} strains have the 4.2 kbp fragment associated with classes E, F, G and I. The remaining two Oak-Ridge strains

Figure 2-3: Autoradiogram of *Hind*III DNA fragments that hybridize to probe p8AS-1 (*het-6V*) from Panama and Oak-Ridge standard strains (left), and selected strains from the Louisiana population. Fragment patterns were placed into classes as indicated across the top of the figure. Size standard at right is lambda DNA digested with *Hind*III and sizes are in kbp.

PA OR

A F I G H F H F B G B E G B A A C

Table 2-2: Mean size (kbp) of restriction fragments that hybridized to probes p8AS-1 and pECO16M in the representative *het-6V* and *het-6J* RFLP classes, respectively.

RFLP classes at *het-6V*

***HindIII* fragments sizes**

A	5.4
B	15.0
C	5.6
E	4.2
F	5.4, 4.2
G	15.0, 4.2
H	15.0, 3.3
I	7.3, 4.2

RFLP classes at *het-6J*

***EcoRI* fragment sizes**

L	16.3
M	12.2
N	16.3, 12.2
U	16.3, 11.7, 8.7

in class H have a unique 3.3 kbp fragment. All but three Oak-Ridge strains (all of class E which have a single 4.2 kbp fragment) exhibited two restriction fragments. All 13 *het-6^{PA}* strains exhibited only a single restriction fragment hybridizing to p8AS-1. None of the *het-6^{PA}* strains had either of the 4.2 kbp or 3.3 kbp fragments, which typifies *het-6^{OR}* strains.

RFLPs at *het-6J*

To examine RFLPs associated with *het-6J*, DNA from *N. crassa* strains was digested with *EcoRI* prior to hybridization with the pECO16M, (Figure 2-2). pECO16M contains an insert of about 4.9 kbp that includes *rnr-1*, the large subunit of ribonucleotide reductase, and *het-6J* incompatibility function has been localized to *rnr-1* by transformation assays (Smith *et al.*, unpublished). There are no *EcoRI* recognition sites within pECO16M, and for this reason the enzyme was selected for analysis of RFLP associated with *het-6J*. Hybridization patterns with pECO16M were examined in 28 Louisiana strains and the *het-6^{OR}* and *het-6^{PA}* standard strains. These RFLP patterns were placed into four distinct classes, L, M, N, and U, as given in Table 2-2. Representatives of these RFLP classes are shown in Figure 2-4. Primarily the presence or absence of two fragments defines these classes. Class L has one fragment of about 16.3 kbp, class M has a single fragment of about 12.2 kbp and class N has both of these fragments. A single strain, 4494 was exceptional in having fragments of about 16.3, 11.7 and 8.7 kbp in size. The numbers of strains with restriction fragment patterns in classes L, M, N, and U were

9, 4, 14 and 1, respectively. Again, there is a good correlation between these RFLP patterns and *het-6* allelic designations based on partial diploid analyses. All but one of the strain in class L was *het-6*^{PA}. All strains in class M were *het-6*^{OR}. All but two strains in class N was *het-6*^{OR}. However, both *het-6*^{PA} and *het-6*^{OR} standard strains had a single fragment of 16.3 kbp and were placed into class L. Another observation about the hybridization patterns when pECO16M was used as a probe is that the autoradiographic intensity of fragments varied substantially between strains, and among fragments of DNA from the same strain (data not shown). This difference in intensity could result from differences in either the quantity or quality of the target DNA used or in the copy number of these sequences in the genome.

Figure 2-4: Autoradiogram of *Eco*RI DNA fragments that hybridize to probe pECO16M (*het-6J*) from Panama and Oak-Ridge standard strains (left), and selected strains from the Louisiana population. Fragment pattern classes are indicated across the top. *Hind*III digested lambda DNA size standard is shown at right with sizes given in kbp.

PA OR

L L U N N M L L N N N

PCR-RFLP analysis of *het-6V* and *het-6J*

Two primer sets, 6VP3 / 6VP5 and 6JP6 / 6JP11, that gave consistently good quantities of PCR product were selected for use in PCR amplification of DNA within *het-6V* and *het-6J*, respectively. PCR products were digested with restriction enzymes and subjected to gel electrophoresis. Primer sets and restriction enzymes were selected initially that differentiated between the PA and OR standard strains. The position of primer pairs in *het-6V* and *het-6J* are given in Figure 2-2. PCR-RFLPs analysis of *het-6V* was carried out with 38 Louisiana strains and with 39 strains for *het-6J* and these PCR-RFLP profiles are given in Table 2-1.

The *het-6V* PCR amplification products were approximately 1.1 kbp in size for all of the *N. crassa* strains tested and for the two standard strains. Each 6VP3 / 6VP5 PCR product was digested with *Mbo*I. *Mbo*I digestion patterns of the 6VP3 / 6VP5 PCR products could be placed in one of two general classes which corresponded to either the PA standard strain with a fragment of just under 1 kbp, or the OR standard strain which produced two co-migrating fragments in agarose gels of about 0.5 kbp (Figure 2-5). Sequence analysis of *het-6V*^{OR} had identified four restriction sites for *Mbo*I within the region defined by primers 6VP3 and 6VP5 and the position of these is illustrated in Figure 2-2 (Smith *et al.*, unpublished). Based on these positions of *Mbo*I restriction sites, we would expect that only the two larger fragments (each of approximately 0.5 kbp) would be visible on the gel and the remaining three fragments would be too small to detect under our electrophoresis conditions. The larger fragment size characteristic of Panama strains may indicate the absence of the three right-most *Mbo*I sites (Figure 2-2);

Figure 2-5: 6VP3/6VP5 PCR products digested with *Mbo*I. At left is molecular size standard in kbp. Strain numbers are given across the top and Panama (PA) and Oak-Ridge (OR) standard strains are shown at right.

digestion of the remaining *Mbo*I site near primer 6VP3 would generate a fragment of approximately 1 kbp. Among the 38 Louisiana strains tested, 15 had the 1 kbp “PA” fragment, and 19 had 0.5 kbp fragment as in the OR standard strain. Four strains (4469, 4479, 4481 and 4494) were observed to have two distinguishable fragments (Figure 2-5). These are designated in Table 1 as being either “PA & OR”, if the two fragments are of the same size as those in PA and OR strains, or “O”, for “other”, in the case of 4479 which has a unique PCR-RFLP profile at *het-6V*.

The approximate size of *het-6J* PCR products using primers 6JP6 and 6JP11 was 1.5 kbp for all strains. To resolve the multiple, small fragments that resulted when these PCR products were digested with *Msp*I, polyacrylamide/agarose gel electrophoresis was used. Electrophoresis of *Msp*I digested *het-6J* PCR products gave two distinctive patterns for Panama and Oak-Ridge standard strains (Figure 2-6). As expected from sequence data (Smith *et al.*, unpublished), *Msp*I digested PCR product from the Oak-Ridge standard strain produced four visible fragments of about 580, 330, 285 and 273 bp in size. Additional expected smaller fragments of less than 100 bp were not resolved under our electrophoresis conditions. PCR product of the Panama standard strain produced fragments of about 600, 300, 270 and 125 bp in size. The 125 bp fragment is not evident in Figure 2-6. Since the undigested Panama PCR product was about 1.5 kbp and these four *Msp*I fragments add up to 1355 kbp, we infer that an additional fragment(s) of less than about 100 bp was not observed using our electrophoresis conditions. Among the 39 isolates tested, 15 had fragment patterns that were indistinguishable from that of the Panama standard strain, and 20 were identical to that of the Oak-Ridge standard strain.

Four strains (4471, 4472, 4479 and 4497) produced fragment patterns that differed from those of both the Panama and Oak-Ridge standard strains. These four isolates each produced fragment patterns similar to the Oak-Ridge standard except that only three fragments were visible; the 285 bp fragment was not evident in these strains (Figure 2-6). Because the undigested PCR products for these four strains was of equivalent size to the standard strains, we infer that this missing fragment is due to additional *MspI* sites and not to deletion of DNA. The *het-6J* PCR-RFLP patterns for these four strains are designated as “O”, for “other”, in Table 2-1.

In all cases, the PCR-RFLP markers for *het-6V* and *het-6J* were correlated to each other and to functional *het-6* allelism based on partial diploid analysis. All strains identified as *het-6J^{PA}* or *het-6J^O* had the *het-6V^{PA}* or *het-6V^O* marker and were *het-6^{PA}* based on partial diploid analysis. All strains identified as *het-6V^{OR}* were also *het-6J^{OR}* and were functionally *het-6^{OR}* based on partial diploid analyses.

Figure 2-6: 6JP11/6JP6 PCR products digested with *Msp*I. Fragments patterns for OR and PA standard strains are shown at left along with representatives from the Louisiana population sample. Molecular size standard (bp) is indicated at right.

Discussion

The objectives of this study were to investigate genetic variation and distribution of alleles at *het-6V* and *het-6J* based on molecular markers in conjunction with a functional assay of *het-6* incompatibility based on partial diploid analysis. In doing this, we also initiated a study of a natural population of *N. crassa* using non-self recognition genes as genetic markers. To our knowledge, this is the first study to use molecular determination of non-self recognition genes to measure genetic variation in a fungal population.

With the genetic markers of non-self recognition used in this study, which include *mat*, *het-6* function, RFLPs and PCR-RFLPs at both *het-6J* and *het-6V*, we were able to distinguish at least 18 unique genotypes in the 33 Louisiana strains. A previous study by Mylyk (1976) used the partial diploids technique (Perkins, 1975) for ascertaining incompatibility alleles at six *het* loci, *het-5*, *het-6*, *het-7*, *het-8*, *het-9*, *het-10*. His analysis of 15 isolates from three Louisiana populations of *N. crassa* revealed that all but two of these strains contained at least one allelic difference at the loci studied. The increased incidence of identical genotypes in our study could reflect the limited area of the genome studied or that our collection was of conidiating masses and may therefore represent clonal dispersal of genets. A second method of examining non-self recognition in fungal population is based on pairwise confrontations of strains to test for “vegetative incompatibility”. If two strains produce a line of demarcation (barrage zone) at these colony confluences, they are said to be vegetative incompatible. Barrage can be identified as either lines of pigment deposition or clear zones. Barrage formation occurs in many, or perhaps all, filamentous fungi and has found widespread application in the study of fungal

populations (Anagnostakis *et al.*, 1986; Kohn *et al.*, 1991; Smith *et al.*, 1994). However, the ease with which barrages can be identified often depends on environmental (culture) conditions. The genetic basis of barrage formation has, in most cases, not been determined, but is underway for several ascomycetes including *Podosporea anserina* (Bégueret *et al.*, 1994), *N. crassa* (Griffiths and Rieck, 1981; Micali and Smith unpublished) and *Cryphonectria parasitica* (Cortesi and Milgroom, 1998).

Electrophoretic markers have been used extensively in the study of fungal populations (review in McDonald and McDermott, 1993), including *Neurospora* populations. Allozyme markers were used to detect variation in strains of *N. intermedia* from natural populations of four different geographic sites (Spieth, 1975). The principal result was a pattern of high level of genetic variation, which were detected within and between local populations, consistent with expectations for an outbreeding species with long range dispersal. Other studies have surveyed the distribution of plasmids among *Neurospora* species (Debets *et al.*, 1994; Arganoza *et al.*, 1994). Based on these studies, horizontal transfer of plasmids appears to occur frequently in nature by heterokaryosis or by transient hyphal fusion events. The efficiency of plasmid transmission can be affected by incompatibility mechanisms involving either *het* genes or mating-type alleles. Incompatibility caused by known *het* genes in some cases prevents or slows horizontal plasmid transmission among *N. crassa* strains (Debets *et al.*, 1994).

We found that RFLPs at both *het-6V* and *het-6J* can be used as naturally occurring genetic markers. The plasmid probes pECO16M (*het-6J*) and p8AS-1 (*het-6V*), detected length polymorphisms and possibly copy number polymorphisms, in *EcoRI* digests and *HindIII* digests of genomic DNA, respectively. Louisiana strains have been subdivided

into at least four classes at *het-6J* and eight *het-6V* classes based on these restriction fragment patterns. Multiple restriction fragments were evident in 15 of 18 OR strains examined for *het-6V* RFLPs, including the standard strain. This is surprising since there are no *HindIII* sites internal to *het-6V* based on digests of the p8AS-1 probe DNA (data not shown). OR strains were characterized as having one fragment of 4.2 kbp (classes E, F, G, and I) or 3.3 kbp in size (class H). The *HindIII* fragment from which p8AS-1 was subcloned was also about 4.2 kbp in size. We hypothesized that the 4.2 and 3.3 kbp fragments, and not the additional fragments in OR strain, carry the OR incompatibility function. The additional fragments of 1.5, 5.4 (which are also found in PA strains) and 7.3 kbp appear to represent an additional region of DNA within the *N. crassa* genome that has sequence identity to *het-6V*. This raises the possibility that *het-6V* may be duplicated in the genome. This hypothesis is now being tested by analysis of *het-6V* fragment inheritance patterns in controlled crosses.

Fourteen of 28 Louisiana strains examined for RFLPs at *het-6J* also exhibited two fragments of about 16.3 and 12.2 kbp in size. Similarly sized fragments were evident in strains with single hybridizing bands. Again, since there are no *EcoRI* sites in the probe pECO16M DNA, two fragments in these strains are consistent with a duplicated segment for which sequence identity occurs outside of *het-6J* in the *N. crassa* genome. RFLP data with genomic DNA was usually, but not always, correlated with functional allelism at *het-6* based on partial diploid analysis. The RFLP characters, which we examined, are expected to be closely linked to, but somewhat independent from internally encoded allelic determinant. The length variations can be due to loss or gain of restriction enzyme recognition sites that flank the incompatibility genes, or to insertions or deletions within

hybridizing fragment. A diversity of fragment sizes could also be achieved by intrafragment recombination. Relatively low *het-6J* RFLP diversity was evident in the Louisiana sample. For example, 14 of 28 isolates are classified in class “N” at *het-6J*. Similarly for *het-6V*, 9 of 30 isolates are classified in class “A”. This should be considered as a minimum RFLP diversity at *het-6J* and *het-6V* since we used only a single restriction enzyme to evaluate RFLPs at each locus and because only fragments separated by more than 1 mm on the autoradiogram were considered to be different.

To develop more precise genetic markers at *het-6J* and *het-6V*, we used a PCR-based method. The effectiveness of the PCR primer sets was evaluated for differentiating between OR and PA at *het-6J* and *het-6V*. The primer sets 6VP3/6VP5 and 6JP6/6JP11 were shown to amplify DNA segments that were polymorphic in restriction enzyme sites (Figure 2-5 and 2-6). When digested with *Mbo*I, the 6VP3/6VP5 PCR product yielded four distinct genotypes. Two genotypes designated *het-6V*^{OR} (19 isolates) and *het-6V*^{PA} (15 isolates) were identical to Oak-Ridge and Panama alleles, respectively. A third genotype *het-6V*^{PA&OR}, was evident in three isolates (4469, 4481 and 4494) as having both Panama and Oak-Ridge fragments. A fourth genotype, in isolate 4479, is characterized as *het-6V*^O (Table 2-1), having two fragments that were unique in size in this population sample (Figure 2-4). Two observations indicate that the samples that yield two PCR-RFLP fragments are not artifacts due to mixed cultures or to mixed DNA samples during PCR. First, in strains producing two *het-6V* PCR-RFLP fragments, only a single fragment was evident for PCR-RFLP analysis of *het-6J*, for which we used the same DNA stock solutions for PCR amplifications. If the cultures or DNA samples yielding the *het-6V* PCR-RFLP fragments were of mixed composition, we would expect to see two *het-6J*

diagnostic patterns. Second, in repeated experiments, the two fragments were evident for *het-6V* in these same strains. This indicates that the two *het-6V* PCR-RFLP fragments were not likely due to target DNA contamination or to errors in setting up the PCR amplifications. The observation of two fragments does, however, corroborate our hypothesis that *het-6V* may be duplicated in the genome of *N. crassa*.

One possible explanation for the four strains with two *het-6V* PCR-RFLP fragments is that both copies are amplified during the PCR. That all four strains are functionally *het-6^{PA}* may indicate that the OR copy is silenced or is recessive to the PA copy. A well studied example of silencing and duplication of non-self recognition genes is found in the mating type of *Saccharomyces cerevisiae*. The mating type of *S. cerevisiae* can be either *a* and α , as determined by two completely dissimilar DNA sequences at the *MAT* locus (Glass and Lorimer, 1991). Both *a* and α cell types contain a silent copy of the *a* sequence at the *HMR* locus and of the α sequence at the *HML* locus. Functional mating-type is conferred by the sequence (either *a* or α) that is present at the *MAT* locus; the *a* and α sequences present at *HMR* and *HML* loci are not transcribed and do not contribute to the mating characteristics of the cell (Klar *et al.*, 1981).

6JP6/6JP11 PCR products were examined after digestion with *MspI* and polyacrylamide electrophoresis. Analysis of *MspI* digested PCR products revealed one of three patterns for all 39 *N. crassa* isolates tested. The PCR-*MspI* patterns were designated *het-6J^{OR}* (20 isolates) and *het-6J^{PA}* (14 isolates). A third pattern, *het-6J^O* was found in four isolates, 4471, 4472, 4479, and 4497. One of our initial objectives was to identify strains from nature that have a different combination of alleles at *het-6J* and *het-6V* than those combinations found in the OR and PA standard strains. Such strains would

provide an opportunity to determine whether both, one or the other of *het-6J* and *het-6V* has heterokaryon incompatibility function. However, strains that are OR/PA or PA/OR for *het-6J/het-6V* appear to be absent from this population sample. For this reason, strains that are of the genotype *het-6J^O* are of interest. The *het-6J^O* genotype obtained for strains 4471, 4472, 4479, and 4497 is most similar to *het-6J^{OR}*, however all four strains are functionally *het-6^{PA}*. A functional incompatibility assay, using *het-6J^{OR}* as transforming DNA would determine whether *het-6J* and *het-6V* have incompatibility function.

Alternatively, the *het-6J^O* genotype may be a new allele that is functionally distinct from both the OR and PA counterparts at *het-6J*. Evidence suggests that multiple alleles exist at *het-c* and *het-8* in *N. crassa* (Howlett *et al.*, 1993; Saupe and Glass, 1997). Multi-allelic loci that are involved in nonself recognition during mating have been characterized in plants (Clarke and Newbigin, 1993) and fungi (Schulz *et al.*, 1990).

The molecular markers of *het-6J* and *het-6V* developed in this study provide a rapid means of evaluating allelic constitution at *het-6* in population samples of *N. crassa*. Using these and other molecular markers of *het* genes, as they become available, should reveal more about the process of genetic variation and function of *N. crassa het* genes and increase our understanding of the molecular basis of non-self recognition as a general biological phenomenon.

Chapter 3

**Molecular markers of heterokaryon incompatibility genes at *het-6*
in worldwide *Neurospora* species and other fungi.**

Introduction

The genus *Neurospora* has been extensively studied since Shear and Dodge first described it in 1927. *Neurospora* can be divided into three heterothallic, five homothallic and at least one pseudohomothallic species based on the sexual cycle. Cytological studies on most of these species have been published: heterothallic species *N. crassa* (McClintock, 1945; Singleton, 1953; St. Lawrence, 1954; Phillips, 1967; Barry and Perkins, 1969; Gillies, 1972), *N. intermedia* (Barry, 1966), and *N. sitophila* (Fincham, 1949; Dodge *et al.*, 1950); the pseudohomothallic *N. tetrasperma* (Dodge *et al.*, 1950); and the homothallic species, *N. terricola* (Nelson and Backus, 1968), *N. africana*, *N. dodgei*, *N. galapagosensis* and *N. lineolata* (Raju, 1977). Heterothallic species require interaction of two strains that differ in mating type (*A* or *a*), to complete the sexual cycle (Shear and Dodge, 1927; Dodge, 1935; Sansome 1946). Meiosis followed by a single mitotic division results in the production of eight haploid ascospores by heterothallic species. Mycelia of homothallic and pseudohomothallic species are “self-fertile” (Glass and Smith, 1994). Homothallic species normally produce eight ascospores that result from nuclear fusion, meiosis and a single mitotic division. Pseudohomothallic species, also referred to as “secondarily homothallic”, produce four ascospores per meiosis, each containing two

nuclei, usually of opposite mating type. Pseudohomothallic individuals maintain the heterokaryotic condition in which *mat-A* is carried by one nucleus and *mat-a* is carried by the other, and are therefore self-fertile.

Of the eleven, well characterized *Neurospora* species, the heterothallic species *N. crassa* has been the most widely used in genetic studies. Two other closely related species, *N. sitophila* and *N. intermedia*, can be sexually crossed to *N. crassa* to produce viable progeny. *N. tetrasperma* is another close relative of *N. crassa*, with the striking difference that it is homothallic. These four species produce abundant orange conidia and occur in warm, humid climates, with a special preference for colonizing the sites of recent fires (Perkins, 1975).

Despite extensive laboratory based experiments, there are few population studies of *N. crassa* and its relatives. This is surprising since an extensive collection of *Neurospora* isolates from nature is available. Perkins (1976) and others have made a worldwide collection of over 3900 isolates of *Neurospora* species from tropical and subtropical regions and these are available through the Fungal Genetic Stock Center (FGSC). These isolates have been collected from over 500 sites (Perkins *et al.*, 1988).

In Chapter 2 of this thesis, the distribution and number of alleles at *het-6* were investigated in a local *N. crassa* population by using partial diploid analysis and genetic markers of *het-6J* and *het-6V* based on PCR and RFLPs. In this chapter, I use RFLP markers to make a preliminary assessment of genetic variation at *het-6J* and *het-6V* in *N. crassa* from the worldwide collection, in other *Neurospora* species and in other more distantly related fungi. The RFLP technique was chosen for this study since it provides a very efficient and sensitive method for obtaining genetic markers that has proven to be

applicable to many different kinds of organisms (Bos, 1996). Most importantly, in RFLP analysis no previous knowledge about DNA sequence of samples to be analyzed is necessary. The first objective of this chapter was to compare and contrast genetic variation at *het-6* in this sample of *N. crassa* from throughout its worldwide range with that of the local Louisiana population examined in Chapter 2. Data are presented here for *N. crassa* collected at 13 sites throughout the tropical Americas, 2 sites in Africa and 7 sites in tropical Asia (Figure 3-1). The second objective of the chapter was to study genetic variation at *het-6J* and *het-6V* in closely related heterothallic, homothallic and pseudohomothallic species of *Neurospora* along with other diverse ascomycete and basidiomycete fungi. It is hypothesized that, to avoid self-incompatibility, pseudohomothallic strains will be homozygous at *het* loci or will be heterozygous with one copy being non-functional. It is also hypothesized that heterokaryon incompatibility will be restricted to a phylogenetically restricted group of species. We expect that the distribution of *het* gene sequences should reflect this restricted distribution.

Figure 3-1: Distribution of *N. crassa* sampling sites. Strains from tropical Americas (13 sites), Africa (2 sites) and Asia (7 sites) were obtained and analyzed.

Methods and Materials

Strains and culture conditions

The worldwide *N. crassa* strains used in this study are listed in Table 3-1 and are available from the Fungal Genetic Stock Center (FGSC, Department of Microbiology, University of Kansas Medical School, Kansas City, Kansas). Sampling points for these strains is shown in Figure 3-1. Strains representing other *Neurospora* species were also obtained from the FGSC and are listed in Table 3-3. Cultures were grown and maintained on 1× Vogels salts (Vogel, 1964), 1.5% sucrose, 1.5% agar, and 1× growth supplements, where necessary (Davis and de Serres, 1970) at room temperature (22°-24°C). Genomic DNA from other species of Ascomycete and Basidiomycete fungi was obtained from M. L. Smith (Table 3-4).

DNA isolation, hybridization and RFLP analysis

DNAs were isolated by the method of Oakley *et al.* (1987) and quantity and quality of DNA was determined after electrophoresis in 0.7% agarose gels and ethidium bromide staining by visual examination over a long-wavelength ultraviolet light source.

Genomic DNA extracts were digested with the restriction endonucleases, *EcoRI* or *HindIII* according to the conditions recommended by the manufacturer (Gibco/BRL, Burlington, ON). Digested DNAs were subjected electrophoreses in 0.7% agarose gels with 1× Tris-acetate/EDTA buffer at 1 V/cm for 16 to 17 hours. Agarose gels were stained with ethidium bromide and photographed over UV light. Capillary transfer of DNA fragments from gels to nylon membranes (Hybond-N⁺, Amersham, England) was

done according to the Southern (1975) method. Probe DNAs were labeled either by nick translation (Bethesda Research Laboratories, Gaithersbourg, MD) or by the random primer method using T7 Quick Prime (Pharmacia, Baie d'Urfe, P.Q.). Prehybridization and hybridization conditions (60°C) and washes were done according to the membrane manufacture's recommended procedure. Hybridization signals were visualized following exposure to Kodak Bio-Max-1 film at -80°C for 1 to 4 days. Fragment mobility was measured as the migration distance from the bottom of the loading well in the agarose gel to the center of the hybridization signal. Molecular size of each fragment was estimated by comparison with the molecular size standard of *HindIII*-digested phage lambda DNA (Boehringer Mannheim).

Table 3-1: *Neurospora crassa* strains and Oak-Ridge and Panama standard strains used in this study and their associated *het-6V* and *het-6J* RFLPs.

FGSC Strain Numbers & <i>mat</i> ¹	Origin	RFLP Patterns ²	
		<i>het-6V</i>	<i>het-6J</i>
<u><i>N. crassa</i> strains</u>			
429 <i>a</i>	Puerto Rico	F	N
430 <i>A</i>	Adiopodoume, Ivory Coast	G	nt ³
851 <i>A</i>	Costa Rica	B	L
852 <i>A</i>	Costa Rica	B	L
961 <i>A</i>	Liberia	A	M
1130 <i>a</i>	Panama Canal Zone	A	L
1131 <i>A</i>	Panama Canal Zone	A	L
1132 <i>a</i>	Panama Canal Zone	A	L
1133 <i>a</i>	Panama Canal Zone	A	L
1165 <i>a</i>	Panama	A	L
1824 <i>A</i>	Lahore, Pakistan	E	L
1945 <i>A</i>	Florida	A	N
2220 <i>A</i>	Louisiana	B	L
2222 <i>A</i>	Louisiana	A	N
2224 <i>a</i>	Louisiana	B	L
2225 <i>A</i>	Texas	A	L
2227 <i>A</i>	Louisiana	H	L
2229 <i>A</i>	Louisiana	B	L
2712 <i>a</i>	Bombay, India	I	L
3199 <i>A</i>	Louisiana	B	L
3201 <i>A</i>	Texas	B	N
3210 <i>A</i>	Louisiana	B	L
3212 <i>A</i>	Louisiana	G	N
3223 <i>A</i>	Louisiana	H	N
3225 <i>a</i>	Texas	B	L
3226 <i>A</i>	Texas	H	N
3227 <i>A</i>	Louisiana	A	L
3359 <i>a</i>	Andhra Pradesh, India	C	L
3360 <i>A</i>	Andhra Pradesh, India	C	L
3693 <i>A</i>	Colonia Paraiso, Puerto Rico	F	N
3885 <i>A</i>	Mt. Wilson, Claifornia	F	N
3943 <i>a</i>	Louisiana	B	L
3968 <i>A</i>	Florida	A	L
3969 <i>A</i>	Florida	A	O
3970 <i>A</i>	Florida	G	O
3972 <i>A</i>	Florida	H	N
3973 <i>A</i>	Florida	A	N
3975 <i>a</i>	Florida	B	P

[Continued on next page...]

[...Table 3-1 continued]

4704A	Dacca, Bangladesh	E	N
4705A	Brazil	F	N
4707a	Bas Quartier, Haiti	A	O
4708A	Berard, Haiti	A	N
4710a	Carrefour Dufort, Haiti	A	L
4711A	Haut Diquini, Haiti	A	L
4712a	Leogane, Haiti	A	L
4713A	Merger, Haiti	G	L
4714A	Pescail, Haiti	A	L
4716A	Puilboreau Mt., Haiti	A	N
4717A	Tamil Nadu, India	D	L
4719A	Tamil Nadu, India	J	L
4720A	Tamil Nadu, India	C	P
4722a	Tamil Nadu, India	J	S
4723A	Penang, Malaya	I	U
4725A	Penang, Malaya	I	R
4726A	Penang, Malaya	B	V
4728a	Penang, Malaya	K	T
4730A	Venezuela	A	N
4819a	Bouanza, Congo	B	Q
4820a	Loubomo, Congo	B	R
4821a	Makaba, Congo	J	M
4822A	Madingo, Congo	A	M
4823A	Libreville, Gabon	A	L
4824A	Carrefour Mme.Gras, Haiti	A	L
4825A	Tiassale, Ivory Coast	A	N
4827A	Tiassale, Ivory Coast	A	M
4828a	Asikro, Ivory Coast	A	N
4829a	Foro-Foro, Ivory Coast	A	M
4830A	Golikro, Ivory Coast	E	M
4831a	Grabiokoko, Ivory Coast	F	M
4832a	Eremankono, Ivory Coast	E	nt
4833A	Ibogue, Ivory Coast	A	L
4834A	Issia, Ivory Coast	A	M
4835A	N'Douci, Ivory Coast	A	L
4837A	Sakota, Ivory Coast	F	M
5359A	Penang, Malaya	nt	Q
5910A	Digitima Creek, Guyana	A	L
5914A	Torani Canal, Guyana	A	L
6202A	Jaco, Costa Rica	A	L
6203A	Agudas, Costa Rica	B	L
6205a	Agudas, Costa Rica	B	L

[Continued on next page...]

[...Table 3-1 continued]

6206A	Esterillo Este, Costa Rica	E	nt
6208A	Esterillo Este, Costa Rica	G	M
6211A	Jaco, Costa Rica	B	L
6212A	Covolar, Costa Rica	H	M
6240A	Maripasoula, French Guiana	A	S
6488A	Klong Rangist, Thailand	K	T
6490A	Khao Eto, Thailand	J	T
6634A	Yucatan, Mexico	A	M
6638A	Yucatan, Mexico	A	L
6688A	Tamil Nadu, India	E	L
7547A	Trinidad	A	nt
7552A	Venezuela	A	nt
7553a	French Guiana	A	nt
7556a	Brazil	A	nt
<u>Standard Strains</u>			
74-OR23-IVA	(Oak-Ridge standard)	F	L
RLM58-18a	(het-6 ^{PA} standard)	A	L

¹mat = mating type, ²RFLP patterns are classed into groups (Table 3-2), ³nt = not tested.

Results

RFLPs at *het-6V* in *N. crassa* strains

Genomic DNA of *N. crassa* from 22 geographic regions and the standard strains, 74-OR23-IVA (*het-6^{OR}*) and RLM58-18 (*het-6^{PA}*) were digested with *Hind*III. The digested DNAs were subjected to electrophoresis, Southern blotted and then hybridized with radiolabeled p8AS-1, which contains the entire *het-6V^{OR}* open reading frame (for a map of the p8AS-1 insert, please see Figure 2-2, Chapter 2). RFLPs at *het-6V* for 93 *N. crassa* strains are presented in Table 3-1. The fragment patterns produced by *Hind*III permitted the placement of strains into eleven classes, designated A through K (Table 3-1 and Figure 3-2). The numbers and sizes of hybridizing DNA fragments that characterized these classes are given in Table 3-2. Forty-one strains were classed as A, seventeen as B, three as C, one as D, six as E, six as F, five as G, five as H, three as I, four as J and two as K. The Oak-Ridge and Panama standard strains were placed into classes F and A, respectively. The distribution of these classes at all sampling points is shown in Figure 3-3. It is apparent from this figure that in some geographical regions, a single RFLP class is detected while multiple classes were evident in other regions. For example, from the Panama Canal Zone all five *N. crassa* fall into class A. On the other hand, the nine strains examined from Costa Rica were classed into five classes, A, B, E, G, and H. Some specific fragments were found in several strains of different geographic origin. For example, the 4.2 kbp fragment was found in strains from the regions of Costa Rica, India, Brazil, Pakistan, Bangladesh, Florida, Louisiana, Haiti, Puerto Rico, Malaya, California and Ivory Coast.

Figure 3-2: Autoradiogram of *Hind*III DNA fragments that hybridize to probe p8AS-1 (*het-6V*) from worldwide collection of *N. crassa*. Fragment patterns were placed into classes and representatives of each class are shown as indicated across the top. Molecular size standard (λ) fragments from top to bottom are 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0 kbp.

D I B H A B A B A E A A A A A B B G F λ J K C C

Table 3-2: Number and mean size (kbp) of restriction fragments in different RFLP classes from a worldwide collection of *N. crassa* strains. Classes at *het-6V* and *het-6J* are based on fragments that hybridized to probes p8AS-1 and pECO16M, respectively.

RFLP classes at *het-6V*

***HindIII* fragment sizes**

A	5.4
B	15.0
C	7.3
D	4.8
E	4.2
F	5.4, 4.2
G	15.0, 4.2
H	15.0, 3.3
I	7.3, 4.2
J	7.3, 5.4
K	14.0, 7.3

RFLP classes at *het-6J*

***EcoRI* fragment sizes**

L	16.3
M	12.2
N	16.3, 12.2
O	17.2, 12.2
P	17.2, 9.4
Q	9.8, 4.4
R	6.6, 4.4
S	16.3, 8.7, 4.4
T	8.7, 4.4
U	16.3, 11.7, 8.7
V	9.0, 4.4

Figure 3-3: Distribution of RFLP markers at *het-6J* and *het-6V* in worldwide *N. crassa* collection. (*Note: The Louisiana site includes RFLP data from Chapter 2 and the additional Louisiana strains given in Table 3-1).

RFLPs at *het-6J* in *N. crassa* strains

RFLP patterns at *het-6J* from 87 strains of *N. crassa* from 22 geographical regions and the *het-6^{OR}* and *het-6^{PA}* standard strains were examined. The genomic DNA of strains was digested with *EcoRI*, subjected to agarose gel electrophoresis, Southern blotted and probed with pECO16M. pECO16M contains *het-6J^{OR}* incompatibility function as described in Chapter 2. *het-6J* restriction fragment patterns were placed into 11 classes, L, M, N, O, P, Q, R, S, T, U and V, as given in Table 3-1 and Figure 3-4. In addition, the numbers of fragments and fragment sizes that define these classes are given in Table 3-2. Forty-one strains were placed in class L, twelve in class M, eighteen in N, three in O, two in P, two in Q, two in R, two in S, three in T, one in U and one in V. Both Panama and Oak-Ridge standard strains had a single fragment of about 16.3 kbp and were placed into class L.

We did not find RFLPs in some geographical regions. For example, five strains from Panama Canal Zone were all in class L. However, multiple RFLP classes were found within other regions. For example, four different classes L, N, O and P were evident in seven strains from Florida. The 16.3 kbp fragment was most common as it was found in sixty-two strains obtained from twelve different geographic regions, some of which were geographically distant.

RFLPs at *het-6V* and *het-6J* in *Neurospora* species

RFLPs at *het-6V* and *het-6J* were also examined in 21 strains representing ten *Neurospora* species (not including *N. crassa*). Table 3-3 presents the numbers and sizes of hybridization fragments for *het-6V* and *het-6J*, respectively. The *het-6J* and *het-6V*

fragment patterns in these strains differed from those identified in *N. crassa*, with some exceptions. For instance, *het-6V* RFLP patterns in *N. sitophila* strains 6673, 6802 and *N. tetrasperma* strains 965, 6228 were the same as in *N. crassa* class E, *N. lineolata* 1910 had the same pattern as *N. crassa* class C and *N. discreta* 6794 was identified to *N. crassa* class B. Similarly, at *het-6J*, *N. dodgei* had the same pattern as *N. crassa* strains in class L. At *het-6V*, little variability was observed within *N. sitophila* and *N. tetrasperma*. Also, all but one *N. sitophila* strain were identical for *het-6J* RFLP patterns. Identical restriction fragment patterns were observed for three out of six *N. intermedia* strains and in two out of four *N. tetrasperma* (Table 3-3). Relatively less variation in fragment sizes was evident within species and more variation was evident between species at both *het-6V* and *het-6J*. Each species had between one and four fragments that were unique to that species. Generally, *N. terricola*, *N. galapagosensis*, *N. discreta*, *N. sitophila*, *N. intermedia*, *N. africana*, *N. sublineolata*, *N. tetrasperma*, *N. dodgei* and *N. lineolata* formed ten separate groups, which correlated with the ten species. However, some fragments appear to be common among strains that represent different species. For example, *N. terricola* at *het-6J* showed the same restriction pattern as a strain (6228) from *N. tetrasperma*.

Hybridization of *het-6V* to other fungal DNAs

The results of Southern hybridizations of *HindIII* digested genomic DNAs with ³²P labelled p8AS-1 are given in Table 3-4. Autoradiographic signals were evident in DNAs only from members of the family Sordariaceae.

Figure 3-4: Autoradiogram of *Eco*RI DNA fragments that hybridize to probe pECO16M (*het-6J*) from worldwide collection of *N. crassa*. Fragment patterns were placed into classes and representative examples of each class is indicated at the top of the figure. Molecular size standard (λ) fragments from top to bottom are 23.1, 9.4, 6.6, 4.4, 2.3 and 2.0 kbp.

O Q N T V R U S P L L N L L L L L λ L M L

Table 3-3: Mean size (kbp) of restriction fragments that hybridized to probes p8AS-1 (*het-6V*) and pECO16M (*het-6J*) in *Neurospora* species.

Species	FGSC & <i>mat</i> ¹	Origin	<i>Hind</i> III fragment(s)	<i>het-6V</i>	<i>Eco</i> RI fragment(s)	<i>het-6J</i>
<i>N. sitophila</i>	417A	Unknown	4.8, 4.2		17.2, 16.3, 6.6	
	2216A	Unknown	4.8, 4.2		17.2, 16.3, 8.7	
	6673 <i>a</i>	Brazil, South America	4.2		17.2, 16.3, 8.7	
	6802A	Robina, Australia	4.2		17.2, 16.3, 8.7	
<i>N. africana</i>	1740h ²	Nsukka, Eastern Nigeria	10.0, 4.8		5.3, 4.0	
<i>N. sublineolata</i>	5508h	Iriomote, Okinawa, Japan	17.0, 8.0		5.5, 3.7	
<i>N. dodgei</i>	1692h	Luquillo Mtns., Puerto Rico	10.0		16.3	
<i>N. terricola</i>	1889h	Spring Green, Wisconsin	8.0		8.7, 4.4	
<i>N. galapagosensis</i>	1739h	Isla Santa Cruz, Galapagos- Islands	10.0		7.8, 4.0	
<i>N. lineolata</i>	1910h	near Lahore, Pakistan	7.3		6.6, 4.0	
<i>N. intermedia</i>	1754 <i>a</i>	Nanking, China	9.8, 7.3		10.9, 4.4	
	962 <i>a</i>	Nanking, China	9.8, 7.3		10.9, 4.4	
	2540 <i>a</i>	Nanking, China	9.8, 7.3		10.9, 4.4	
	1882A	Queensland, Australia	9.8		12.2, 4.4	
	3996A	Queensland, Australia	9.8		8.7, 4.4	
	1830A	Queensland, Australia	9.8, 7.3		12.2, 8.7, 3.7	
<i>N. tetrasperma</i>	350 <i>a</i>	Unknown	4.2, 3.7		16.3, 6.6, 4.4	
	1270A	Unknown	4.2, 3.7		16.3, 6.6, 4.4	
	965PS ³	Liberia	4.2		16.3, 10.9, 4.4	
	6228PS	Gabon	4.2		8.7, 4.4	
<i>N. discreta</i>	6794A	Ivory Coast	15.0		16.3, 10.9, 4.8	

¹*mat*= mating-type, ²homothallic, ³Pseudohomothallic.

Figure 3-5: Restriction fragments in *Neurospora* species hybridizing to *N. crassa*

het-6J^{OR} probe pECO16M (top) and *het-6V^{OR}* probe p8AS-1 (bottom).

Fragment sizes (kbp) are given at left. Species names are given at the top of figure. Strain designations are given at top of both panels.

	<i>N. sitophila</i>			<i>N. africana</i>			<i>N. sublineolata</i>			<i>N. dodgei</i>			<i>N. terricola</i>			<i>N. galapagosensis</i>			<i>N. lineolata</i>			<i>N. intermedia</i>			<i>N. tetrasperma</i>			<i>N. discreta</i>		
	417	2216	6673	6802	1740	5508	1692	1889	1739	1910	1754	962	2540	1882	3996	1830	350	1270	965	6228	6794									

17.2

16.3

12.2

10.9

8.7

7.8

6.6

5.5

4.8

4.4

4.0

3.7

	417	2216	6673	6802	1740	5508	1692	1889	1739	1910	1754	962	2540	1882	3996	1830	350	1270	965	6228	6794
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17.0

15.0

10.0

9.8

8.0

7.3

4.8

4.2

3.7

Table 3-4: DNA hybridization (present = +, absent = –) of *het-6V* probe DNA to DNAs of other fungal species.

Subdivision	Family	Species	Hybridization to <i>het-6V</i>
Ascomycotina ¹	Sordariaceae	<i>Galasinospora tetrasperma</i>	+
		<i>Neurospora crassa</i>	+
		<i>Neurospora discreta</i>	+
		<i>Neurospora galapagosensis</i>	+
		<i>Neurospora intermedia</i>	+
		<i>Neurospora pannonica</i>	+
		<i>Neurospora sitophila</i>	+
		<i>Neurospora terricola</i>	+
		<i>Neurospora tetrasperma</i>	+
		<i>Sordaria brevicollis</i>	+
		<i>Sordaria macrospora</i>	+
		<i>Sordaria sclerogenia</i>	+
	Trichocomaceae	<i>Aspergillus nidulans</i>	—
		<i>Aspergillus niger</i>	—
	Saccharomycetaceae	<i>Saccharomyces cerevisiae</i>	—
		<i>Schizosaccharomyces pombe</i>	—
	Hypocreaceae	<i>Fusarium avenaceum</i>	—
		<i>Fusarium oxysporum</i>	—
		<i>Fusarium proliferatum</i>	—
		<i>Fusarium moniliforme</i>	—
		<i>Fusarium solani</i>	—
	Sclerotiniaceae	<i>Trichoderma reesei</i>	—
		<i>Botrytis cinerea</i>	—
		<i>Sclerotinia sclerotiorum</i>	—
Basidiomycotina	Schizophyllaceae	<i>Sclerotinia rolfsii</i>	—
		<i>Schizophyllum commune</i>	—
	Ustilaginaceae	<i>Ustilago bullata</i>	—
		<i>Ustilago hordei</i>	—
		<i>Ustilago kulleri</i>	—
		<i>Ustilago maydis</i>	—

¹Taxonomy based on Hawksworth, Sutton and Ainsworth, 1983.

Discussion

RFLPs in genomic DNA were detected within *N. crassa* and between the *Neurospora* species examined in this study. The first objective of the present study was to compare and contrast genetic variation in *N. crassa* strains collected worldwide to those collected from a Louisiana population at the *het-6* locus. For the purpose of this study, population refers to a group of individuals that are present in a restricted area or a single field. A “site” refers to strains collected within a limited geographic region, like Haiti. The *N. crassa* population from Louisiana was subdivided into eight classes at *het-6V* and four classes at *het-6J* (refer to Chapter 2, Table 2-1). *N. crassa* strains, which were collected from different regions of four continents (Asia, Africa, North and South America), were subdivided into eleven classes at both *het-6J* and *het-6V* (Table 3-1). Although no site specific RFLPs are evident, some bands were predominantly present in certain regions, suggesting some geographical differentiation with respect to structure of the distribution of markers. For example, Class C was relatively common in strains collected at the India sites, compared to other sites, where it was only found in Louisiana (Figure 3-3). Similarly, Class A appeared to be common in West African sites and among eleven sites in Americas (Figure 3-3), but was not detected in strains from Asia. At the continental level, the strains collected from Asia were classed into six classes at *het-6V* and seven classes at *het-6J*. Interestingly, the fragment of 4.2 kbp at *het-6V* and 16.3 kbp at *het-6J* were observed in all strains from Asia with the exception of the strains from Thailand, which had neither the 4.2 kbp nor 16.3 kbp fragment (Table 3-1 and 3-2). Based on the

correlation of the presence of the 4.2 kbp fragment and *het-6*^{OR} function (Chapter 2) we can hypothesize that the strains are predominantly OR at *het-6*.

The strains from Africa could be placed into six *het-6V* classes, all having the 5.4 kbp fragment in common and five classes at *het-6J* with 12.2 kbp fragment which was found in strains from Liberia, Ivory Coast and Congo (Table 3-1 and 3-2). Thus, although there is much genetic similarity among these tropical sites, there appears to exist some geographical differentiation (Figure 3-3).

In some regions, there seems to be higher level of fragment polymorphism than in other regions. For instance, *N. crassa* strains from Asia and Africa seem to be more polymorphic than those from other regions. This is suggested by the observation that, almost without exception, any two strains from the same site in Asia exhibit a difference in at least at one of the *het-6V* or *het-6J* markers (Figure 3-3). On the other hand, variation appears to be lower among strains from the Americas with respect to the number of alleles at *het-6V* and *het-6J* loci. For example, all forty-six strains from different regions of Americas fell into just six classes at *het-6V* and six classes at *het-6J*. Surprisingly, there is a striking degree of uniformity in restriction fragment patterns among strains from Florida, Louisiana and Texas (Table 3-1, Figure 3-5). The most striking example of this uniformity in the Americas is in the Panama region, where five strains were placed into one class for each locus, class A at *het-6V* and class L at *het-6J*. This relatively low level of variation at *het-6V* and *het-6J* in the Americas suggests lower genetic variability in the new world compared to Asia and Africa for this species. This may mean that a relatively small number of *N. crassa* genotypes were introduced from the old world into the Americas at some point in time. Similar observations were also made with *Cryphonectria parasitica*

based on nuclear RFLPs, where there is evidence for a low degree of genetic polymorphism in the United States compared to China, where *C. parasitica* is thought to be native (Milgroom and Lipari, 1993). In other fungi, populations may exhibit significant genetic variation even over short distances, sometimes of only a few hundred meters (McDonald and McDermott, 1993; David and Capy, 1988). A pattern of small, but significant genetic differences between *N. crassa* populations in the Americas is fully compatible with the idea that geographic distance among populations is not necessarily correlated with genetic distance.

The second objective of this chapter was to survey the genetic variation at *het-6J* and *het-6V* in closely related *Neurospora* species along with other ascomycete and basidiomycete fungi. A number of approaches for studying genetic variation within and between species have been used. Kohn *et al.* (1988) used RFLPs in nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) of *Sclerotinia* species. Their analysis of 42 isolates from *Sclerotinia* populations revealed that most variation in restriction fragments of rDNA and mtDNA were between species and they could apply these markers as taxonomic characters in comparing species. In Figure 3-5 of this thesis it is evident, that most of the variation in restriction fragment lengths was between rather than within *Neurospora* species. For example, the three species, *N. sitophila*, *N. intermedia* and *N. tetrasperma*, for which there were multiple strains examined, formed distinct groups based on these markers. The exception to this, however, is seen when comparing strains (1692 and 1739) of *N. dodgei* and *N. galapagosensis* at *het-6V* and, strains (1889 and 6228) of *N. terricola* and *N. tetrasperma* at *het-6J*, which have the same RFLP patterns (Figure 3-5). This indicates either that these species are closely related or that there are constraints

on these sequences, such that sequence divergence is not possible. The other interesting pattern was observed between heterothallic *N. sitophila* and pseudohomothallic *N. tetrasperma* at *het-6J*. Pseudohomothallic individuals produce heterokaryotic self-fertile ascospores. Pseudohomothallics can complete the sexual cycle and produce progeny more rapidly than heterothallics because there is no need for each germling to find a sexually compatible partner. However, pseudohomothallic species of a genus typically occur as exceptions among species that are heterothallic, and may retain heterothallic features (Raju and Perkins, 1994). For example, when *N. tetrasperma* is resolved into separate *A* and *a* components, each homokaryotic strain retains the ability to make trichogynes which respond to the opposite mating-type, just as occurs in heterothallic species (Raju and Perkins, 1994). In this study, we hypothesized that, to avoid self-incompatibility, pseudohomothallic strains will be homozygous at *het* loci or will be heterozygous with one copy being non-functional. From our preliminary observations, it was difficult to determine whether one or both of these hypothetical possibilities occurred. In homokaryotic strains (350*a* and 1270*A*) of the pseudohomothallic species, *N. tetrasperma*, multiple fragments were evident where both *het-6V* and *het-6J* used as probes. In general, fewer fragments were evident in the heterokaryotic strains of *N. tetrasperma* (965PS and 6228PS) than in the homokaryotic strains. This seems counterintuitive, because it is expected that more fragments should be present in heterkaryotic pseudohomothallic strains. It is hypothesized that heterothallic species are ancestral to pseudohomothallics rather than vice versa (White-house, 1949; Dodge *et al.*, 1950; Burnett, 1956; Raju and Perkins, 1991). Based on the RFLP data presented here, this hypothesis can neither be supported, nor rejected.

Finally, the *het-6V* probe as a molecular marker revealed that this sequence is restricted to a phylogenetically closely related group, the Sordariaceae (Table 3-5). This preliminary examination of the distribution of *het-6V* sequence supports the placement of *Neurospora* and other genera within the Sordariaceae.

This study used molecular genetic markers of heterokaryon incompatibility genes to reveal information about the genetic structure of *Neurospora* populations. The limited number of genetic markers used suggests that *N. crassa* genomes have diverged on the four continents. It is clear that many important questions in *Neurospora* population remains, but the necessary tools are now available to address them with increasing levels of confidence. *Neurospora* not only has an illustrious past in the study of genetic but also a promising future. It should be a useful organism for which genetic studies at the molecular and population levels will progressively produce convergent and interacting data and so help us to understand the genetic mechanisms of evolutionary processes, since evolution basically is progressive change in the genetic composition of populations.

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