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Molecular and Functional Analyses of Incompatibility Genes at *het-6* in a Population of *Neurospora crassa*

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Mir-Rashed, N., Jacobson, D. J., Dehghany, M. R., Micali, O. C., and Smith, M. L. 2000. Molecular and functional analyses of incompatibility genes at *het-6* in a population of *Neurospora crassa. Fungal Genetics and Biology* 30, 197–205. Two closely linked genes, *un-24* and *het-6,* associated with the *het-6* heterokaryon incompatibility functional haplotype were examined in 40 *Neurospora crassa* strains from a Louisiana sugarcane field. Partial diploid analyses were used to determine that half of the strains were functionally Oak Ridge (OR) and half were non-OR and indistinguishable from the standard Panama (PA) form. PCR-based markers were developed to identify polymorphisms within both *un-24* and *het-6.* Two common forms of each gene occur based on these molecular markers. Rare forms of both *un-24* and *het-6* were identified as variants of the non-OR form by a DNA transformation assay. The heterokaryon incompatibility function of haplotypes, based on partial diploid analyses, was perfectly correlated with the PCR-based markers at both loci. This correlation indicates that the two loci are in severe linkage disequilibrium in this population sample and may act as an incompatibility gene complex. Southern hybridizations using OR- and PA-derived cloned probes from the region that spans *un-24* and *het-6* showed that the apparent absence of recombination in this ~25-kbp region is associated with low levels of overall sequence identity between the PA and OR forms. © 2000 Academic Press

Index Descriptors: fungi; non-self-recognition; recombination; disequilibrium; gene complex.

Non-self-recognition in filamentous fungi occurs during both the asexual and sexual stages of the life cycle (reviews in: Glass and Kuldau, 1992; Leslie, 1993; Bégueret *et al.*, 1994; Coppin *et al.*, 1997; Worrall, 1997). One well-studied form of non-self-recognition is the heterokaryon incompatibility system in *Neurospora crassa.* This system is controlled by at least 10 heterokaryon incompatibility (*het*) loci and the mating-type locus (*mat*) (Perkins, 1988). A difference at any one of these loci prevents the stable growth of heterokaryotic hyphae during the vegetative phase of the life cycle but does not interfere with the limited heterokaryosis associated with sexual reproduction. *het* gene differences in *N. crassa* restrict transmission of cytoplasmic elements between individuals (Debets *et al.*, 1994) and prevent parasitism of unfertilized strains by nuclei of the same mating type (Debets and Griffiths, 1998). These potentially important functions would suggest that allelic variation at *het* loci would be maintained within populations by balancing selection (review in Klein *et al.*, 1998; Hartl *et al.*, 1975; Nauta and Hoekstra, 1994). Recently, the occurrence of balancing selection at the *het-c* locus in *N. crassa* was supported by observations that allelic forms of *het-c* are maintained across genera within the Sordariaceae, that relatively large numbers of nonsynonymous base substitutions occur within the *het-c* specificity region, and that approximately equal numbers of

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RFLPs, indicative of the three allelic forms of *het-c,* occurred in a field population of *N. crassa* from Louisiana (Wu *et al.,* 1998). Based on the balancing selection model, we would expect (1) approximately equal frequency of allelic forms at other *het* loci and (2) linkage equilibrium at independently functioning *het* loci in field populations.

In this study, we examined heterokaryon incompatibility at *het-6* using a functional assay and molecular genetic markers within a population sample of *N. crassa* from Louisiana. Mylyk (1975) identified *het-6* on the left arm of linkage group II (LG II,[1] Fig. 1) by partial diploid analyses using the translocation strains *T(II → VI)P2869* and *T(II → III)AR18.* The *het-6* heterokaryon incompatibility phenotype is associated with at least two closely linked genes within the 270-kbp translocated region of *T(II → III)AR18* (Smith *et al.,* 2000b). The two genes have been named *un-24* and *het-6* and together form the haplotype we will refer to as the *het-6* region. Both genes possess at least two allelic variants, Oak Ridge (OR) and Panama (PA), named for the wild-type strain of origin. Superscripts of these symbols are used to denote wild-type alleles at a locus. The Oak Ridge haplotype will be referred to as OR and the Panama haplotype as PA. *un-24* encodes the large subunit of ribonucleotide reductase (Smith *et al.,* 2000a), while *het-6*^{OR} putatively encodes a protein of 680 residues of unique sequence (Smith *et al.,* 2000b). DNA transformations with the OR allele of either gene (*het-6*^{OR} or *un-24*^{OR}) into PA, but not OR, spheroplasts results in a loss of viability of transformants (Smith *et al.,* 2000b).

Our objectives in this study were threefold. The first was to develop molecular markers to easily discriminate alleles at *un-24* and *het-6.* The second was to examine the relationship between these molecular markers and incompatibility function. The third was to estimate the number and frequencies of alleles at *un-24* and *het-6* and to determine whether combinations of alleles at the two loci provide additional heterokaryon incompatibility specificity.

METHODS AND MATERIALS

Strains, Media, and Culture Conditions

A sample of 54 *Neurospora* isolates was collected as masses of conidia from separate distinct colonies on burned sugarcane stubble in a 5-ha field near Franklin, Louisiana. Each isolate was cultured, purified, and identified to species using standard methods (Perkins and Turner, 1988) and has been deposited in the Perkins collections (Nos. P4448–P4501), which is curated at the Fungal Genetics Stock Center (FGSC, Department of Microbiology, University of Kansas Medical School, Kansas City, KS). Forty of the 54 isolates were identified as *N. crassa* (Table 1). The *het-c* allele was determined for 36 of these strains previously as Oak Ridge, Panama, or Groveland, by correlation of RFLP patterns to known reference strains (Wu *et al.,* 1998). The *het-c* locus is ~12 MU centromere proximal to *het-6* on Linkage Group II (Fig. 1). Other strains used in this study were *het-6*^{PA} standard strains, RLM58-18 and C2(2)-1 (Smith *et al.,* 1996), *het-6*^{OR} standard strains, 74-OR23-1VA (FGSC 2489) and C9-2 (Saupe *et al.,* 1996), the RFLP mapping strains (FGSC Nos. 2225, 4450–4488), and translocation strains *T(II → VI)P2869* (FGSC 1828*A* and 1829*a*) and *T(II → III)AR18* (FGSC 2643*A* and 2644*a*). *N. crassa* strains were cultured with either solid or liquid Vogel's medium and crosses were performed using Synthetic Cross Medium and standard methods (Davis and de Serres, 1970; Perkins, 1986). For partial diploid analyses, a wild strain and the translocated strain of opposite mating type were coinoculated to a 13 × 100 mm tube containing 2.5 ml Synthetic Cross Medium. 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 an equally spaced grid pattern on a single 9-cm-diameter petri dish containing minimal medium with sorbose (1%), fructose (0.05%), and glucose (0.05%) substituted for sucrose to restrict colony size and supplemented with tyrosine (0.5 g/L) and phenylalanine (0.2 g/L) to encourage inhibited partial diploid progeny to produce brown pigment. The entire plate was placed at 60°C for 40–45 min, incubated for 2 days at 34°C, and examined under a dissecting microscope (magnification up to ×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). Only inhibited colonies developed brown pigment, usually after 3 to 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

[1] Abbreviations used: LG II, linkage group II; OR, Oak Ridge; PA, Panama.

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determined by visual approximation after agarose gel electrophoresis and staining with ethidium bromide over a long-wavelength ultraviolet light source.

DNA Transformations

Transformation of spheroplasts derived from macroconidia was by the method of Royer and Yamashiro (1992). The strains C9-2 and C2(2)-1 were used in transformation experiments as *het-6*^{OR} and *het-6*^{PA} functional haplotype standards, respectively. Approximately 1 μg of DNA of various constructs of the hygromycin-resistance vector pCB1004 (Carroll *et al.,* 1994) was mixed with 100 μl of a spheroplast suspension (about 8.0×10^7 spheroplasts/mL). Transformants were selected on medium with a final hygromycin-B (Calbiochem, La Jolla, CA) concentration of 250 U/mL.

RFLP Analysis

Genomic DNA was digested separately with *Eco*RI and *Hin*dIII (Gibco/BRL, Burlington, Ontario), fractionated by electrophoresis in 0.7% agarose with a 1× Tris–acetate/EDTA buffer at 1 V/cm for 16 to 17 h, and transferred to nylon membranes (Hybond N, Amersham, UK) by the method of Southern (1975). DNA probes were labeled with [α-^{32}P]dCTP by nick translation (Bethesda Research Laboratories, Gaithersbourg, MD) or by the random primer method using T7 Quick Prime (Pharmacia, Baie d'Urfe, Québec). Prehybridization and hybridization conditions were according to the membrane manufacturer's recommended procedure. Autoradiography was performed with Kodak Bio-Max-1 film at −80°C for 1 to 4 days. Probe DNAs included p8AS-1, pECO16M, and pA3 (Fig. 2). p8AS-1 has a ~2.4-kbp *Apa*I/*Sma*I insert that contains *het-6*^{OR} (Smith *et al.,* 2000b). pECO16M has a ~4.8-kbp *Eco*RI/*Mbo*I insert that contains *un-24*^{OR} (Smith *et al.,* 2000a). pA3 was prepared for this study by cloning a ~11-kbp *Hin*dIII fragment from strain RLM58-18 (*het-6*^{PA} standard strain) into the vector pUC118 (Sambrook *et al.,* 1989). pA3 contains all of *un-24*^{PA} and about 1.7 kbp of the 5′ end (~84%) of the *het-6*^{PA} coding region.

Polymerase Chain Reaction (PCR) Amplification

Oligonucleotides used in PCR reactions were synthesized (Molecular Genetics Laboratories at Carleton University, Biology Department) based originally on the DNA sequence of Oak Ridge alleles at *het-6* (GenBank Accession No. AF206700) or *un-24* (GenBank Accession No. AF171697). The *het-6*^{PA} sequence was subsequently determined (GenBank Accession No. AF208542; Smith *et al.,* 2000b). In addition, the sequence of 72.8 kbp of OR-background DNA that includes *un-24* and *het-6* was recently made available at MIPS (contig b2a19, Munich Information Center for Protein Sequences, *N. crassa* genome project, http://www.mips.biochem.mpg.de/desc/neurospora/). Combinations of primer pairs and restriction enzymes were tested with standard OR (74-OR23-1V) and PA (RLM58-18) strains to identify PCR-based markers at both loci. For allele typing at *het-6,* primers 6VP3 (5′-CGGTAACCTGTTCAGCT-3′) and 6VP5 (5′-CCCGCTAAGCCAAGGAGTCC-3′) were used (Fig. 2). For *un-24,* primers 6JP6 (5′-GTGCGGGCTTAACCGCTG-3′) and 6JP11 (5′-CTCCGGATGAGGTTGCCG-3′) were used.

For *het-6,* PCR was carried out in a final reaction volume of 50 μl and included about 100 ng *N. crassa* genomic DNA, 200 μM each dNTP, 10 μM each of primers, 1.5 mM $MgCl_2$, 1.25 units *Taq* DNA polymerase, and 1× *Taq* buffer (Gibco BRL, Burlington, ON). The PCR program was 30 cycles of: 15 s at 95°C (denaturation), 30 s at 48°C (annealing), and 2 min/1 kbp DNA at 72°C (extension). Amplified products were analyzed by gel electrophoresis in 1.5% agarose, 1× TAE (0.04 M Tris–acetate, 1 mM EDTA), with and without digestion with *Mbo*I restriction enzyme.

For *un-24,* the PCR reaction volume was 50 μl and the conditions were 300 nM each primer, 200 μM each dNTP, 2.27 units Expand DNA polymerase mix (Boehringer Mannheim, Laval, QC), 1× Expand reaction buffer, 1.5 mM $MgCl_2$, and between 60 and 200 ng of *N. crassa* genomic DNA. The same cycle parameters were used for *un-24* as described for *het-6* except the annealing temperature was changed from 48°C to 58°C. About 2 μg of each *un-24* 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% nondenaturing polyacrylamide gels containing 10% glycerol, 0.5% agarose, and 1× TBE (0.095 M Tris–borate, 2 mM EDTA, pH 8.0). Electrophoresis conditions were 200 V, 15 mA for 10 min, then 180 V, 14 mA for 1 h, followed by 140 V, 10 mA for 2 days, all at 4°C while recirculating the buffer. After electrophoresis the gels were stained with ethidium bromide and photographed.

RESULTS

Partial Diploid Analyses

The translocation strains, hereafter referred to as *T(AR18)* and *T(P2869),* are known to produce partial dip-

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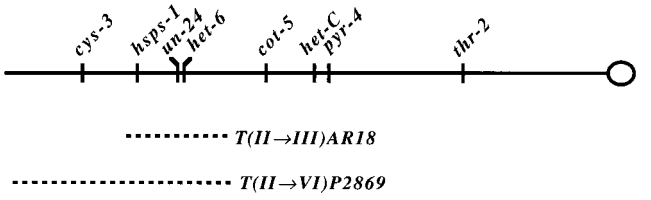

FIG. 1. Selected genetic markers on left arm of linkage group II in *Neurospora crassa* (Perkins *et al.,* 1982). Translocated segments in *T(II → III)AR18* and *T(II → VI)P2869* are indicated below the map; these are the segments duplicated in the partial diploid progeny.

loids for the *het-6*-region (Mylyk, 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 LG II (Fig. 1; Perkins *et al.,* 1982). Both *T(P2869)* and *T(AR18)* are OR for the *het-6* region (Mylyk, 1975). One third of the viable progeny from a cross involving either translocation-bearing strain and a normal sequence strain will have the LG II region of the translocation duplicated. If the parent wild-type and translocation strains differ in the *het-6* region, progeny carrying this duplication will be self-incompatible, i.e., inhibited growth accompanied by a brown discoloration (Mylyk, 1975; Perkins, 1975).

In the first round of partial diploid analyses the 40 Louisiana strains were crossed to *T(P2869)* and progeny were examined for self-incompatibility. Twenty Louisiana wild-type strains were observed to produce 20–42% inhibited progeny. It was tentatively concluded from this that alleles in the *het-6* region of each of these wild-type strains were functionally different from the OR alleles in *T(P2869).* However, since *T(P2869)* duplicates a large segment of LG IIL, it is possible that inhibited progeny may be caused by heterozygosity not only in the *het-6* region, but also at any as yet undescribed *het* loci centromere distal to the *het-6* region. To confirm that the *het-6* region 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 LG IIL segment around the *het-6* region. All partial diploids involving *T(AR18) (DpAR18)* initially have an inhibited, colonial phenotype even when homozygous at *het-6.* However, *DpAR18* that are heterozygous at the *het-6* region 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 that *DpAR18* cultures be observed daily, for up to 7 days, and compared to *DpAR18* cultures of both homozygous and heterozygous *het-6* haplotypes obtained from crosses with OR and 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 *T(AR18)* were responsible for the inhibited phenotype. From this it was inferred that 20 strains carried the OR haplotype and the remaining 20 strains carried a non-OR haplotype in the *het-6* region (Table 1, where strains designated as functionally non-OR are indistinguishable from the PA standard strain based on this partial diploid assay).

RFLPs in the un-24-het-6 Region

Genomic DNAs of 30 *N. crassa* samples from Louisiana and the OR and PA standard strains were digested with *Hin*dIII. *Hin*dIII does not cut within the *het-6*^{OR} gene. The digested DNAs were subjected to electrophoresis, Southern blotted, and then hybridized with a radiolabeled insert from p8AS-1, containing the entire *het-6*^{OR} open reading frame (Smith *et al.,* 2000b; Fig. 2). Fragments that

TABLE 1

het-6 Function and PCR-RFLP Markers for Louisiana Population Sample and Standard Strains

Strain and *mat*[a]	*het-6* function[b]	PCR-RFLP[c] *un-24*	PCR-RFLP[c] *het-6*
Louisiana strains			
P4448*a*, P4449*A*, P4452*a*, P4468*A*, P4469*a*, P4476*a*, P4481*a*, P4485*A*, P4486*A*, P4489*a*, P4490*A*, P4493*a*, P4494*A*, P4498*a*, P4500*a*, P4501*a*	Non-OR	PA	PA
P4450*a*, P4451*a*, P4453*A*, P4454*A*, P4455*a*, P4456*a*, P4457*a*, P4459*a*, P4463*A*, P4464*A*, P4465*a*, P4467*A*, P4470*a*, P4480*a*, P4483*a*, P4484*A*, P4487*A*, P4491*a*, P4496*a*, P4499*a*	OR	OR	OR
P4471*a*, P4472*a*, P4497*a*	Non-OR	D	PA
P4479*a*	Non-OR	D	D
Standard strains			
RLM58-18*a*	PA	PA	PA
74-OR23-1V*A*	OR	OR	OR

[a] *mat,* mating-type locus.

[b] Based on partial diploid analysis, strains that are not OR at *het-6* are designated "non-OR"; these are indistinguishable from functionally PA forms.

[c] PCR-RFLP designated as OR-like, PA-like, or "D" for different (see Figs. 5 and 6).

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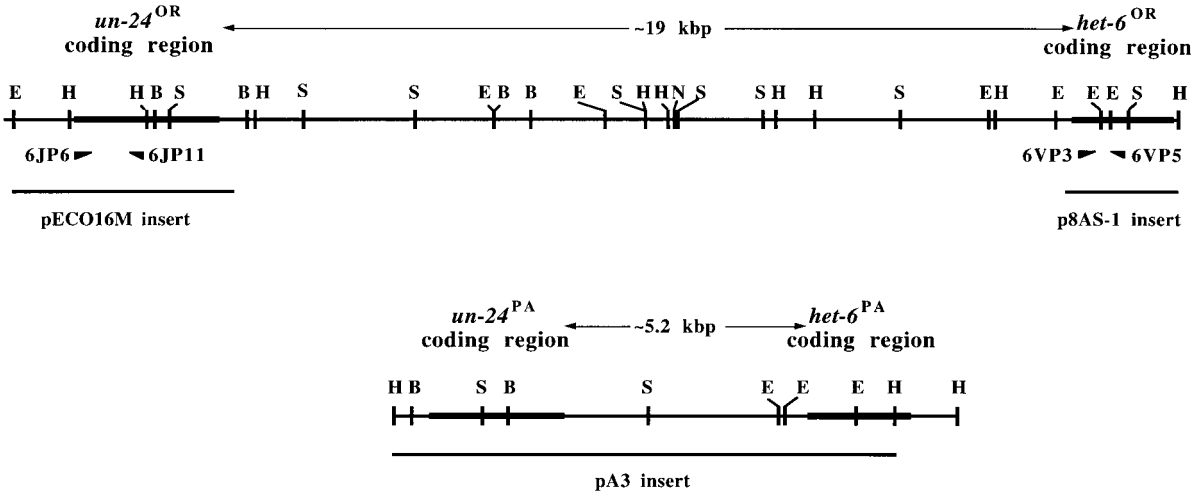

FIG. 2. Restriction site maps of OR (top) and PA (bottom) forms of the *het-6* region. The positions of selected restriction enzyme recognition sites (B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Not*I; S, *Sal*I) are based on restriction site mapping and sequence at the MIPS database (http://www.mips.biochem.mpg.de/desc/neurospora/). The coverage of inserts for plasmids p8AS-1 (contains all of *het-6*^{OR}, dark line at right), pECO16M (contains all of *un-24*^{OR}, dark line at left), and pA3 (contains all of *un-24*^{PA} and most of *het-6*^{PA}) are shown below the respective maps. Annealing positions of primers 6VP3 and 6VP5 are given for the *het-6*^{OR} coding region and for primers 6JP11 and 6JP6 within the *un-24*^{OR} coding region.

hybridized to the *het-6*^{OR} probe were detected in 17 of these 30 isolates and in the *het-6*^{OR} standard strain (Table 1). However, all 32 strains (including both standard strains) hybridized to the *N. crassa pan-2*$^{+}$ gene probe excised from pOKE103 (a gift from J. Grotelueschen and R. Metzenberg) (Fig. 3). The 13 Louisiana strains and the PA standard strain that did not hybridize to the *het-6*^{OR} probe were functionally PA in partial diploid tests. All of the strains that hybridized to the *het-6*^{OR} probe were functionally OR, and all but two had a 4.2-kbp fragment hybridizing to the *het-6*^{OR} probe. The exceptions were two functionally OR strains (P4454 and P4456) that had a fragment of about 3.3 kbp hybridizing to the *het-6*^{OR} DNA probe.

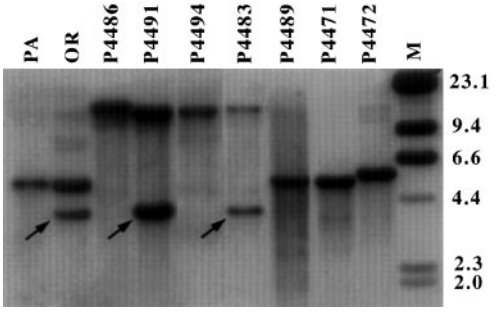

FIG. 3. Autoradiogram of *Hin*dIII DNA fragments from Panama (PA RLM58-18) and Oak Ridge (OR 74-OR23-1V) standard strains and selected strains from the Louisiana population (numbers across top). Fragments that hybridize to *het-6*^{OR} probe are indicated by arrows. Unmarked fragments hybridize to the *pan-2*$^{+}$ probe DNA, included as an internal positive control. DNAs that hybridized to *pan-2*$^{+}$ probe but not to *het-6*^{OR} probe were all from functionally non-OR strains. Size standard (M) at right is λ DNA digested with *Hin*dIII and sizes are in kbp.

To examine RFLPs associated with *un-24*, DNAs from *N. crassa* strains were digested with *Eco*RI, blotted, and hybridized with the 4.8-kbp insert of pEco16M that includes *un-24*^{OR} (Smith *et al.*, 2000a). There are no *Eco*RI recognition sites within this 4.8-kbp insert. Hybridization patterns with pEco16M were examined in 28 Louisiana strains and the 2 standard strains. Each strain yielded a single hybridizing fragment, either of ~9.7 kbp (3 non-OR strains and 17 functionally OR) or ~11 kbp (9 non-OR strains, including the PA standard strain, RLM58-18, and the OR standard strain 74-OR23-1VA).

The construct pA3 contains a ~11-kbp insert of PA-background DNA. By probing this construct with 6JP11/6JP6 and 6VP3/6VP5 PCR products from *un-24*^{PA} and *het-6*^{PA}, respectively, we determined that pA3 contains sequences from both these genes. RFLP mapping data and the MIPS sequence data were used to generate maps of the *un-24–het-6* region for the OR and PA standard strains 74-OR23-1VA and RLM58-18, respectively (Fig. 2). From these maps it is evident that there are major structural differences between the two forms. The distance between *un-24*^{OR} and *het-6*^{OR} is ~19 kbp, while only ~5.2 kbp separate *un-24*^{PA} and *het-6*^{PA}. In addition, a low

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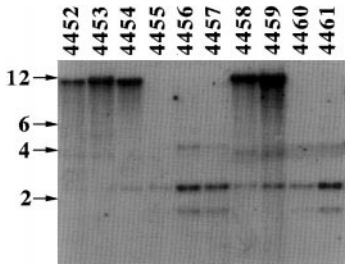

FIG. 4. Autoradiogram of *Hin*dIII-digested DNA from RFLP mapping strains probed with pA3. *het-6*^{PA} strains were FGSCs 4452, 4453, 4454, 4458, and 4459. *het-6*^{OR} strains were FGSCs 4455, 5556, 4457, 4460, and 4461. Positions of size standards (kbp) are given at left.

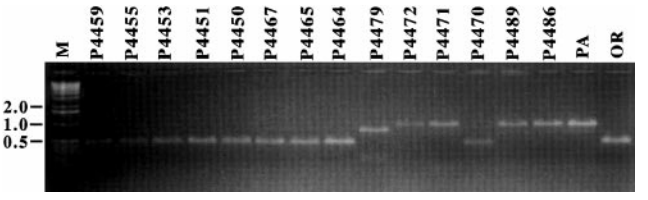

FIG. 5. 6VP3/6VP5 (*het-6*) PCR products digested with *Mbo*I. At left is a 1-kbp molecular size standard (M) in kbp (Gibco/BRL). Strain numbers are given across the top with RLM58-18 (PA) and 74-OR23-1V (OR) standard strains at right.

level of sequence identity was evident between the OR and PA forms of this region based on Southern analysis. In Fig. 4, where pA3 was used to probe RFLP mapping strains, *het-6*^{PA} strains exhibit the expected ~11-kbp fragment, while only a ~1.7-kbp fragment is evident in *het-6*^{OR} strains, aside from a second band of just over 2 kbp which appeared in nearly all lanes of this autoradiograph. RFLP mapping of this polymorphism verified that sequences hybridizing to pA3 in *het-6*^{PA} strains segregate opposite *het-6*^{OR} (Metzenberg and Grotelueschen, 1993).

PCR–RFLP Analysis of het-6 and un-24

To develop molecular markers that distinguish alleles of *un-24* and *het-6* based on internal sequence characteristics we used a PCR-based method. Initially to differentiate between the PA and OR standard strains, two primer sets and restriction enzymes were selected for PCR–RFLP allele typing of *het-6* (6VP3/6VP5 PCR products cut with *Mbo*I) and *un-24* (6JP6/6JP11 PCR products cut with *Msp*I). The positions of primer pairs in *het-6* and *un-24* are given in Fig. 2.

The 6VP3/6VP5 PCR amplification products were approximately 1.0 kbp in size for all 40 *N. crassa* strains tested. *Mbo*I digestion patterns of these PCR products could be placed into one of two general classes which corresponded to either the PA standard strain (a fragment just under 1.0 kbp) or the OR standard strain (two fragments each ~0.5 kbp) (Fig. 5). *het-6*^{OR} contains four *Mbo*I sites in the region flanked by 6VP3 and 6VP5 (Fig. 2). Based on DNA sequence we expect that the two larger fragments (each ~0.5 kbp) would be visible and the remaining three fragments would be too small to detect by the electrophoresis conditions used. The larger fragment size of the *het-6*^{PA} class is due to the absence of all four *Mbo*I sites found in the OR form and a unique site at about 960 bp in the 1020-bp PCR product. Among the 40 Louisiana strains, 19 had the 960 bp *het-6*^{PA} fragment and 20 had the 0.5 kbp *het-6*^{OR} fragments. Strain P4479 had an additional variant of 800 and 200 bp fragments (Fig. 5). This pattern, designated "D" for "different" in Table 1, is interpreted to represent a *het-6*^{PA}-like PCR product with an additional *Mbo*I site.

The size of *un-24* 6JP6/6JP11 PCR products was ~1.5 kbp for all strains. PCR products of *un-24* digested with *Msp*I gave distinct patterns for the PA and OR standard strains (Fig. 6). As expected from sequence data (Smith *et al.*, 2000a), *Msp*I-digested PCR product of *un-24*^{OR} had four visible fragments of 580, 330, 285, and 273 bp. Additional smaller fragments of <100 bp were not resolved under these conditions. PCR products of *un-24*^{PA} produced *Msp*I fragments of 600, 360, 270, and 125 bp (the 125-bp fragment is not seen in Fig. 6). Since the undigested *un-24*^{PA} PCR product was about 1.5 kbp and these

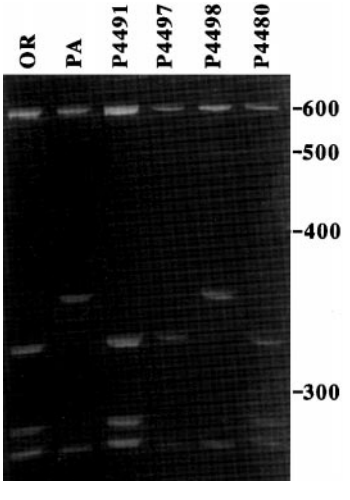

FIG. 6. 6JP11/6JP6 (*un-24*) PCR products digested with *Msp*I. Fragment patterns for OR and PA standard strains are shown at left along with four representatives from the Louisiana population sample. Positions of molecular size standards are given in base pairs at right.

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four *Msp*I fragments add up to 1355 bp, we infer that additional fragments of less than about 100 bp were not visible using these conditions. Among the 40 isolates, 16 had fragment patterns indistinguishable from *un-24*^{PA}, and 20 were identical to *un-24*^{OR}. Four strains (P4471, P4472, P4479, and P4497) produced a fragment pattern that differed from those of both *un-24*^{PA} and *un-24*^{OR} (Fig. 6); this pattern is designated "D" for "different" in Table 1.

Incompatibility Function of un-24^{D} and het-6^{D}

Whether *un-24*^{D} and *het-6*^{D} function as non-OR alleles was tested by DNA transformation assays. Spheroplasts of strains P4471 *un-24*^{D} *het-6*^{PA} *a* and P4479 *un-24*^{D} *het-6*^{D} *a* were transformed separately with each of three plasmids; "plasmid 1" contained the *hyg*^{R} selectable marker alone, "plasmid 2" contained *hyg*^{R} and *un-24*^{OR}, and "plasmid 3" contained *hyg*^{R} and *het-6*^{OR}. As controls, each plasmid was also transformed into the standard strains, C9-2 (*un-24*^{OR} *het-6*^{OR}) and C2(2)-1 (*un-24*^{PA} *het-6*^{PA}). Transformation of P4471, P4479, and C2(2)-1 behaved similarly in these transformation assays; each yielded 100–200 colonies/μg of plasmid 1 DNA and 0 colonies/μg of plasmids 2 and 3. Transformations of C9-2 with each of the three plasmids yielded >80 colonies/μg of DNA. This transformation assay indicates that strains carrying either *un-24*^{D} or *het-6*^{D} are incompatible with the OR-haplotype, and are therefore indistinguishable from the PA alleles at these loci. Since *un-24*^{PA} and *het-6*^{PA} do not have incompatibility activity in transformations (Smith *et al.*, 2000b), and translocations of the PA haplotype for this region are not available, we can not unambiguously determine whether *un-24*^{D} and *het-6*^{D} are compatible with *un-24*^{PA} and *het-6*^{PA}, respectively.

Recombination Frequencies in the het-6 Region

het-6 and *un-24* appear to be inherited as a block; all strains identified by PCR-RFLP as *un-24*^{PA} or *un-24*^{D} were also *het-6*^{PA} or *het-6*^{D} and functionally non-OR, and all strains identified as *het-6*^{OR} were also *un-24*^{OR} and functionally OR. Restriction enzyme maps indicate that *un-24* and *het-6* are separated by ~19 and ~5.2 kbp in OR and PA strains, respectively (Fig. 2). It is unlikely that the physical proximity of these genes, alone, accounts for the observed linkage disequilibrium. In a laboratory cross (Smith *et al.*, 2000b), recombination frequencies in the 30-kbp centromere distal and 40-kbp centromere proximal regions of the *un-24–het-6* gene pair were ~0.1 MU/kbp. We expect, therefore, that the genetic distance between *un-24* and *het-6* is in the range of 0.5 to 2 MU. In our population sample recombination events within about 2.3 kbp between *un-24* and a centromere distal *Eco*RI site may explain the departure from perfect association between RFLP and PCR-RFLP markers in our population sample (above). The *un-24*^{PA} PCR-RFLP fragment is associated with a ~11-kbp *Eco*RI RFLP fragment in 8 strains and the *un-24*^{OR} PCR-RFLP fragment with a ~9.7-kbp *Eco*RI RFLP fragment in 17 strains. Three strains (P4471, P4481, and P4494) have a ~9.7-kbp RFLP fragment associated with the *un-24*^{PA} or *un-24*^{D} PCR-RFLP fragments.

DISCUSSION

The objectives of this study were to investigate genetic variation and distribution of alleles at *un-24* and *het-6* based on molecular markers in conjunction with a heterokaryon incompatibility functional assay based on partial diploid analyses. This is the first study to use a population genetics approach to compare both molecular and functional polymorphisms at a *het* locus. A previous study by Mylyk (1976) used analysis of partial diploids (Perkins, 1975) to ascertain incompatibility alleles at six unlinked *het* loci, *het-5, het-6, het-7, het-8, het-9,* and *het-10.* His analysis of 15 isolates from three different Louisiana populations of *N. crassa* revealed that all but 2 of these strains contained at least one allelic difference at the loci studied. Recently, Wu *et al.* (1998) used restriction fragment patterns to infer that three alleles at *het-c* were in approximately equal frequency in the same Louisiana *N. crassa* population sample examined in this study. We have revealed that this population is also balanced in its representation of two functionally distinct forms of the *het-6*-haplotypes: of the 40 strains examined by partial diploids analysis, 20 were functionally OR and 20 were non-OR, or PA-like. Equal allelic frequency such as this could occur through chance or could indicate that the two forms are maintained in the population by means of a frequency-dependent selection mechanism, as suggested for *het-c.*

We found that functional allelism, based on partial diploid tests, was invariably correlated with the PCR-RFLP alleles at both *un-24* and *het-6* in this population sample. This corroborates data from transformation-based assays that both *un-24* and *het-6* contribute to heterokaryon incompatibility function associated with the *het-6* region (Smith *et al.*, 2000b). Of four possible allelic combinations,

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however, only *un-24*^{OR} *het-6*^{OR} and *un-24*^{PA} *het-6*^{PA} were observed. The two other combinations in the population sample are expected under a model of frequency-dependent selection if *un-24* and *het-6* function independently. This model would predict that strains with rare combinations of alleles at distinct *het* loci would have a selective advantage and that each combination would tend toward approximately equal frequencies. This is clearly not the case. However, linkage equilibrium in the Louisiana population is, in fact, evident for the *het-6*-haplotype and the three *het-c* alleles (Wu *et al.*, 1998; N. L. Glass, pers. comm.). The *het-6–het-c* combinations PA–OR, OR–OR, PA–PA, OR–PA, PA–GR, and OR–GR occur in 7, 7, 7, 7, 4, and 4 strains, respectively.

Taken together, our data suggest that *un-24* and *het-6* act as an incompatibility complex. This is the only example, of which we are aware, where two closely linked genes apparently contribute to a single heterokaryon incompatibility phenotype. Persistence of this incompatibility complex could be due to suppression of recombination in the region or to nonviability of recombinant progeny. In support of the former, there are significant sequence differences between the two forms that may prevent homologous recombination (Figs. 2–4). The distance between *un-24* and *het-6* is about ~19 kbp in the OR form compared to ~5.2 kbp in the PA form. Restriction site maps also differ significantly in the region between the two genes. Finally, overall DNA sequence identity between the two forms is low; aside from *un-24*, there is little or no cross-hybridization between the OR and PA DNAs in the region covered by pA3. It is interesting that at least four predicted genes are located between *un-24*^{OR} and *het-6*^{OR} (MIPS database, http://www.mips.biochem.mpg.de/desc/neurospora/), including the essential regulatory gene *cys-3*. The lack of sequence identity between OR and PA strains in the region covered by pA3 suggests that the genes located between *un-24*^{OR} and *het-6*^{OR} are either highly dissimilar or are located elsewhere in strains carrying the PA form. We are now using this population-based information to characterize the genetic structure of the *het-6*^{PA} region, to define the extent of this recombination block, and to identify PA-specific incompatibility factors in the region.

The molecular markers developed for *un-24* and *het-6* in this study provide a rapid means of evaluating allelic constitution of the *het-6* haplotype in population samples of *N. crassa.* Examining the distribution of these markers in the Louisiana population provides unique insights into the molecular basis of *het-6* incompatibility function. Similar approaches would be useful in understanding non-self-recognition systems in general.

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