How to identify and score genes that confer vegetative (heterokaryon) incompatibility.

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Background

The heterokaryotic condition, first described by Dodge (1927) in *N. tetrasperma*, provided a ready means for determining the dominance of traits expressed in the haploid vegetative phase. Heterokaryons also seemed to offer a novel genetic system, in contrast to diploidy. The advent of recessive auxotrophic mutations in 1941 provided a means of selecting heterokaryons composed of strains with complementing requirements. Early attempts to exploit heterokaryons in *N. crassa* were frustrated by heterokaryon incompatibility (Beadle and Coonradt 1944). Stable heterokaryons could not be obtained if two strains differed in alleles at any of the numerous genes at what came to be called heterokaryon incompatibility (*het*) loci. The mating type genes of *N. crassa* were also found to act as *het* genes: stable heterokaryons could not be formed between strains of opposite mating type. *het* genes at several loci were polymorphic in laboratory stocks (Holloway 1955), and the different genes were all alike in being recognized because they blocked heterokaryon formation. This made it very difficult to enumerate the *het* genes and to map and characterize them individually. Nevertheless, Garnjobst (1953) and Wilson and Garnjobst (1966) succeeded in identifying and mapping *het-c*, *het-d*, and *het-e*.

Studies of heterokaryon incompatibility were greatly facilitated by the discovery that *het* genes result in visible phenotypic abnormalities when they are heterozygous in segmental duplications, (Newmeyer and Taylor 1967, Perkins 1975), and by the ability to create duplications that are heterozygous for only a single *het* locus. Duplications (partial diploids) of known gene-content can be obtained as progeny from crosses heterozygous for insertional or terminal rearrangements (see Figure 2 in Perkins 1997). Use of duplication-generating rearrangements to survey strains from natural populations led to the discovery of additional *het* loci (*het-5* - *het-10*) and revealed that *het* genes are highly polymorphic in wild populations of *N. crassa* (Mylyk 1975, 1976). Because *het* genes result in cellular death and abnormal growth when they are heterozygous in duplications, the term *vegetative incompatibility* came to be used interchangeably with *heterokaryon incompatibility*. Abnormal growth also results following transformation when a *het* allele is introduced into nuclei of a recipient strain with which it is *het*-incompatible (Saupe and Glass 1997).

When two *het*-incompatible strains come together, hyphal fusion is typically followed by closure of septal pores to seal off adjoining cells, and death of the fused cells. Genes at a locus called *het-i* are atypical. Forced heterokaryons between *het-i* and *het-I* initially show normal growth. Then they stop growing because the *het-i* component is eliminated (Pittinger and Brawner 1961, Wilson *et al.* 1999, Wilson and Holden 2001). In strains of genotype *het-C, -d, -e* (typical of OR genetic background), *het-i* nuclei are not eliminated if they exceed a threshold of 70%, but this is not true for strains that are *het-C, -D, -E* (typical of RL genetic background). The erratic growth reported by Jacobson *et al.* (1995) for combinations of the FGSC Wilson-Garnjobst testers in race tubes was apparently due to differences at *het-i* (Wilson and Holden 2001).

Figure 1 (taken from Perkins *et al*.1993) shows genetic map locations of the known *het* loci, relevant markers, and duplicated segments used for analysis.

Most *het* genes are distinguished by locus numbers. *het-c, -d, -e,* and *-i* are exceptions, however. capital and small letters were used to distinguish the first two alleles at these four loci, which were the first *het* genes to be discovered. Multiple alleles and alleles from different sources are

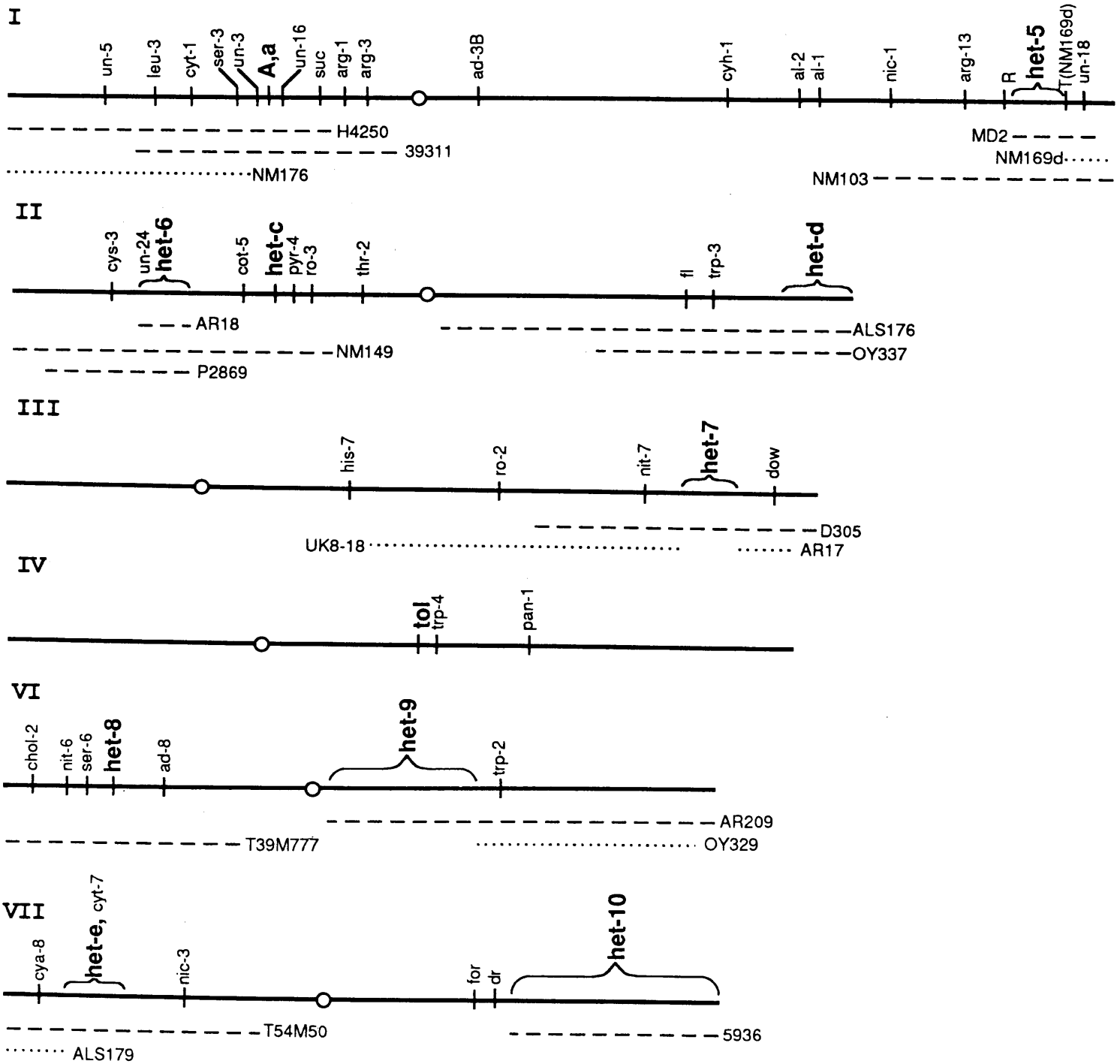

Figure 1. Location of known *het* loci in the *N. crassa* linkage groups. Dashed lines below the linkage groups show the extent of duplications that include each locus and can be used to determine whether incompatible *het* alleles are heterozygous. Dotted lines below the maps show the extent of duplications that do not include the nearby *het* locus and may therefore be useful in determining its map location. For example, in a cross of *translocation MD2 het-5*^{OR} × *normal sequence het-5*^{PA}, one third of viable progeny are duplications. These are heterozyous for the *het-5* alleles and are therefore inhibited, showing that the *het-5* locus is right of the MD2 breakpoint and therefore right of the *R* locus. In a cross of *translocation NM169d het-5*^{OR} × *normal sequence het-5*^{PA}, the duplication progeny are not inhibited, showing that the *het-5* locus is left of the NM169d breakpoint and therefore left of *un-18*.

specified in the conventional way, using superscripts to indicate the allele or strain of origin, for example *het-6*^{PA}. (AD - Adiopodoumé, EM - Emerson, GR - Groveland, OR - Oak Ridge, PA - Panama, RL - Rockefeller-Lindegren.)

Multiple alleles: Multiple *het-c* alleles exist in nature and have also been generated in the laboratory (Saupe and Glass 1997, Wu and Glass 2001). Multiple alleles may also be present at *het-8* (Howlett *et al*. 1993), but the observations on *het-8* could be explained by two alleles at each of two closely linked *het* loci, similar to *het-6*, where the adjoining gene *un-24* (which specifies ribonucleotide reductase) also acts as a *het*-incompatibility locus (Smith *et al.* 2000a, b). Three functional specificities are known for alleles at the polymorphic *het-c* locus: *het-c*^{OR}, *het-c*^{PA}*, and het-c*^{GR}. OR specificity is shown by *het-C* (also symbolized *het-C*^{OR} or *het-c*^{OR}). This allele is present in OR and RL laboratory wild types. GR specificity is shown by *het c*^{GR} (from Groveland, Florida) (Saupe and Glass 1997). PA specificity is shown by *het-c*^{PA} (from Panama) (Saupe and Glass 1997), by laboratory strains *het-c* (Garnjobst 1953) (called *het-c*^{EM} by Saupe and Glass (1997), and by *het-c*^{AD} (from Adiopodoumé, Ivory Coast) (Saupe and Glass 1997).

Suppressors of het-incompatibility: Expression of the vegetative incompatibility associated with mating type requires that an unlinked 'suppressor' gene called *tol* (*tolerant*) be functional (Newmeyer 1970, Jacobson 1992, Shiu and Glass 1999). *tol* is specific for the *het* reaction between *mat A* and *mat a*. It does not suppress incompatibility at any of the other known *het* loci (Leslie and Yamashiro 1997). A partial suppressor of *het-c* vegetative incompatibility called *vib-1* (*vegetative incompatibility blocked*) has been described (Xiang and Glass 2002). Loss-of-function mutations in *vib-1* also suppress incompatibility of *mat* incompatible partial diploids (Xiang and Glass 2004). Mutations that suppress the incompatibility reaction of other *het* genes were reported by Arganoza *et al.* (1994), but those suppressor strains are not available. Modifiers affecting the speed of heterokaryon formation were described by de Serres (1962b).

For reviews of the cell biology and evolutionary biology of heterokaryon incompatibility in Neurospora, see Glass *et al*. (2000) and Glass and Kaneko (2003). Kaneko *et al*. (2006) describe a *het* domain which appears to be present at all loci that are known to mediate vegetative incompatibility. The sequence of this domain may provide a means for identifying additional *het* loci that have not yet been described.

Procedures

Methods of scoring

- *Forced heterokaryons*: Complementing auxotrophic mutations, genes conferring resistance to toxic agents, or recessive genes that limit growth can be used as forcing markers. A simple and usually effective test is to superimpose inocula of the two prospective components at a point on the surface of selective medium using large (75 mm) slants or petri dishes. Freshly grown mycelia or conidia should be used. If stability of the resulting heterokaryon is in question, race tubes may be preferred. Alternatively, successive transfers to fresh slants can be used to determine stability. If defined nuclear ratios are required for initiation of a heterokaryon, conidial suspensions of known density can be pooled, pelleted by centrifugation, and used for inoculation (Pittenger *et al.* 1955). If ability of hyphae to fuse is impaired, a low concentration of one or more of the forcing supplements can be added to the medium to allow limited growth of one of the components (S. Haedo, personal communication; method published in Xiang *et al.* 2002).. A method for testing large numbers of isolates using liquid media was devised by de Serres (1962a).

- *Determining compatibility with OR standards*: Strains are conveniently tested for OR-compatibility by determining whether heterokaryons form on minimal medium with *helper-1* (Perkins 1984) or other OR-compatible helper strains that contain forcing auxotrophic markers. Because mating-type mediated *het*-incompatibility is absent in the helpers, *mat A*, *mat a*, and strains of unknown mating type can all be tested for differences at other *het* loci.. See *How to use helper strains*.

- *Scoring het-i*: Because (*het-I* + *het-i*) heterokaryons grow normally at first but are then unstable owing to loss of one component, scoring is best accomplished by transferring successively to a series of slants or by monitoring growth on race tubes. Incompatibility is apparent when growth falters after 1 to 3 serial transfers (Wilson *et al.* 1999), or after 36 to 48 hours in race tubes.

- *Visual observations*: When strains contain no forcing markers, microscopic examination can be used to determine whether fusion of two strains occurs and whether interstrain fusions are followed by continuing flow or by cellular death. Observations can be made with confidence using a fluorescent dye (FM4-64, FM1-43, or propidium iodine), which clearly marks dead cells apart from cells that are still alive. Evans blue has also been used both by Jacobson *et al.* (1998) and by the Glass laboratory, who now routinely use methylene blue (0.003% final concentration) incorporated into media on plates. The dead hyphal compartments take up the dye and can be visualized without disturbance. The methylene blue affects growth rate of wild type a bit, but does not cause death. Less than 1% cell death is present when wild type is grown on these plates (N. L. Glass, personal communication).

  Methods for live cell imaging of hyphal fusion (Hickey *et al.* 2002) were modified to view incompatibility between strains (D. J. Jacobson, unpublished). The leading edge of a mycelium of each strain is cut and the agar block transferred on a plate of weak medium (0.1× minimal medium) covered with a cellophane membrane. The blocks from strains to be tested should be placed 0.5 – 1.0 cm apart and allowed to grow 2-4 hours or until hyphae have met within the gap between them. The cellophane membrane in this area can be excised and mounted on a slide, stained with a fluorescent dye (see below) under a coverslip. The zone where hyphae from the two colonies meet is examined for cell death, or lack thereof, under epifluorescence illumination with filter sets appropriate for the dye used.

  FM4-64 and FM1-43 (Molecular Probes, Inc. Eugene, Oregon) only enter living cells through endocytosis and subsequently will differentially stain a subset of membranes (Fischer-Parton *et al.* 2000, Read and Hickey 2001, Hickey *et al.* 2004). Upon damage to the plasma membrane, through breakage or programmed cell death, the dye floods into the cell and stains the contents brightly (see Glass and Kaneko 2003 for micrographs). Recommended concentration is 32µM in liquid medium. Propidium iodine is a DNA intercalator, but is excluded from living cells. A 0.15mM solution in liquid medium stains nuclei only in dead or dying cells, whereas living hyphae are not stained at all (Hickey *et al.* 2004).

  If the required optical equipment and reagents are not available, the procedure of Wilson and Garnjobst (1966), described below, can be used. This method, while more laborious, does not employ a dye or depend on fluorescence microscopy.

  "Colonies of the strains to be tested were grown in Petri plates for about 18 hours at 30°C. Small agar blocks were cut from these large colonies at the mycelial frontier and placed mycelium side down on 22 × 30 mm coverslips, previously coated with minimal agar medium, supplemented when necessary. These preparations were incubated in a moist

chamber at either 20° or 30°C until the hyphae of the two strains had intermingled for 1 or 2 hours. Each preparation was then placed on a de Fronbrune oil chamber (Aloe Company) filled with a 15% sucrose solution to keep the hyphae from swelling and bursting.

The preparations were examined with a standard brightfield microscope. Since cells killed by the incompatibility reaction are more dense optically than living hyphae, it is possible to recognize them with 150× magnification. When no incompatibility reactions were observed, the microcultures were reincubated at 30°C for one-half hour to establish protoplasmic flow as the hyphae close to the outside of the coverslip began to grow. It was then generally possible to trace the flow back through the interstrain fusions, proving that the strains were compatible. When necessary, the experiment was repeated until interstrain flow of protoplasm was observed."

- *Heterozygous duplications (partial diploids)*: Testers for particular *het* loci are listed in Table 1, and extent of the duplications used to identify and map each *het* gene is shown in Figure 1.. Characteristics of individual duplication-producing rearrangements and stability of their duplication progeny are described in Perkins (1997). A strain to be tested is crossed with a suitable rearrangement tester. Ascospores are germinated on complete medium or on minimal medium supplemented with tyrosine and phenylalanine. With most duplication-producing rearrangements, one third of viable progeny are duplications. If the duplicated segment is heterozygous for alleles at a *het* locus, these progeny are expected to show inhibited growth and produce dark pigment. The degree of inhibition and intensity of pigmentation differ for different *het* genes, as illustrated in Figure 36 of Perkins *et al.* (2001).

Testers for scoring het-c, -d, -e, and -i by their ability to form stable forced heterokaryons. A table given by Wilson and Holden (2001) lists 22 FGSC *N. crassa* strains that have been completely scored for *het-c*, *-d*, *-e*, and *-i* and that contain forcing markers making them suitable as testers when used as partners for heterokaryon formation. Most of these strains are listed as Wilson-Garnjobst testers in Part VI D of the FGSC catalog. The temperature-sensitive morphological *scot* mutation is present in most of these testers, but it is said not to interfere with scoring at 34°C or below. Because of *het-i*, *scot*, and other possible complicating factors, Jacobson *et al.* (1995) suggest that caution be used when these strains are employed for scoring *het-c*, *-d*, and *-e*.

Testers for creating partial diploids to determine the het alleles present at individual het loci. Table 1 lists reference strains and strains with relevant linked markers, both in normal sequence and in rearrangements capable of generating duplications that include the *het* locus in question.

Screening wild strains for new het loci. Following the procedure adopted by Mylyk (1975), *N. crassa* strains from natural populations are crossed by a series of laboratory strains each known to contain an insertional or terminal chromosome rearrangement that produces duplications covering a different chromosomal region. Progeny are examined for the inhibited growth and reduced fertility that result when *het* alleles are heterozygous. Mylyk screened five wild strains from different populations by crossing them to 15 rearrangements that generate different duplicated segments in nine chromosome arms. Seven of the testers produced a class of inhibited duplications, and six new *het* genes were found and designated *het-5 – het-10*. To select additional wild strains suitable for future screens, see Part V of the FGSC catalog. To choose rearrangements for use in examining previously untested segments, see Perkins (1997) or Perkins *et al.* (2001), where a map shows segments that can be obtained as duplications. See *How to obtain duplications covering known chromosome segments.*

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Table 1. Strains for studying individual *het*-loci of *Neurospora crassa*

Genotype	FGSC No. *mat A*	FGSC No. *mat a*
het-C* and *het-c (IIL)		
Alleles from nature are of three functional specificity types: OR, PA, and GR (Wu *et al.* 1998). The allele historically designated *het-C* (Garnjobst 1953) is *het-c*^{OR}. That designated *het-c* is *het-c*^{GR} (Wu *et al.* 1998; also called *het-c*^{EM} by Saupe and Glass 1997, who showed *het-c*^{AD} also to be type PA). All strains listed here are *het-6*^{OR}.		
het-C (type *het-c*^{OR}) (OR wild types)	2489	4200
het-c (type *het-c*^{GR})	7335	7336
het-C pyr-4	4030	4031
het-c pyr-4	7145	7146
cot-5 het-C	3560	3561
cot-5 het-c	7447	--
cot-5 het-C pyr-4 thr-2	7355	7356
T(IIL→VR)NM149 het-C	3879	3880
T(IIL→VR)NM149 het-c	1483	1482
T(IIL→VR)NM149 het-C pyr-4	--	3136
T(IIL→VR)NM149 het-C ro-3	2011	2012
het-c^{AD} (type *het-c*^{PA})	430	2614
het-c^{AD} *pyr-4 thr-2*	7313	--
T(IIL→VR)NM149 het-c^{AD}	2191	2192
T(IIL→VR)NM149 het-c^{AD} *pyr-4*	7314	7315
het-c^{GR} (type *het-c*^{GR})	2195	1945
T(IIL→VR)NM149 het-c^{GR}	2193	2194
het-d (IIR) (All are *het-C*)		
het-D (RL wild types)	2218	2219
het-d (OR wild types)	2489	4200
T(IIR→VL)ALS176 het-D	3014	2414
T(IIR→VL)ALS176 het-d	3013	2415
T(IIR→IVR)OY337 het-D	7472	7473
T(IIR→IVR)OY337 het-d	3666	3667
het-e (VIIL)		
het-E (RL wild types)	2218	2219
het-e (OR wild types)	2489	4200
T(VIIL→IVR)T54M50 het-E	2603	2604
T(VIIL→IVR)T54M50 het-e	2466	2467
T(VIIL→IVR)T54M50 het-e nic-3	3132	3133
het-i (I or II by linkage to *T(4637) al-1*)		
het-i (ST74A, OR8-1a)	262	988
het-I al-2 nic-1	7343	--
het-i al-2 nic-1	--	7344
het-I T(I;II)4637 al-1; pan-1	7342	--

het-5 (IR)		
het-5^{PA} (Panama CZ30.6)	1131	2190
arg-13 het-5^{PA} (b_{11} × OR)	7345	--
thi-1 ad-9 nit-1 het-5^{PA} (b_{10} × OR)	7348	7349
T(IR→VIR)NM103 het-5^{PA} (b_4 × OR)	7346	7347
het-5^{OR} (OR wild types)	2489	4200
T(IR→II)MD2 het-5^{OR}	3826	3827
T(IR→VIR)NM103 cyh-1 al-1 arg-13 R het-5^{OR}	--	3135

het-6 (IIL)		
All *het-6*^{OR} strains are *un-24*^{OR}. All *het-6*^{PA} are *un-24*^{PA}. Allele *un-24*$^{-}$ (also called *un-24*) is a temperature-sensitive mutant of *un-24*^{OR}. Alleles *un-24*^{OR} and *un-24*^{PA} are not termperature sensitive. Where not specified, the strain is *het-c*^{OR}. Duplications from translocation NM149 include both the *het-c* locus and the *het-6* locus. Whether *het-6* heterozygosity contributes to an incompatible phenotype when NM149 is used as tester can be determined by progeny-testing with translocations AR18 or P2869, which do not cover *het-c*.		
het-6^{PA} (Panama CZ30.6, CZ30.4 [*het-C*?])	1131	1130
het-6^{PA} (2190 is *het-C*^{PA})	2189	2190
het-6^{PA} *arg-12* (b_9 from Spurger P836)	7350	7351
T(IIL→VR)NM149 het-6^{PA} (b_7 from P836)	7352	7353
T(IIL→VR)NM149 het-6^{PA} (Probably *het-C*)	2647	2188
het-6^{OR} (OR wild types)	2489	4200
un-24^{-} *het-6*^{OR} (*un-24*^{OR} mutant)	7354	--
T(IIL→IIIR)AR18 het-6^{OR}	1561	1562
T(IIL→VI)P2869 het-6^{OR}	1828	1829
T(IIL→VR)NM149 het-6^{OR}	3879	3880
T(IIL→VR)NM149 het-6^{OR} *(het-c)*	1483	1482
T(IIL→VR)NM149 het-6^{OR} *pyr-4*	--	3136
T(IIL→VR)NM149 het-6^{OR} *ro-3*	2011	2012
het-7 (IIIR)		
het-7^{LI} (Liberia UA-1)	961	--
het-7^{OR} (OR wild types)	2489	4200
T(IIIR→X;IIIR;VIIL)D305 het-7^{OR}	2139	2140
T(IIIR→X;IIIR;VIIL)D305 het-7^{OR} *dow*	3150	3151
het-8 (VIL)		
het-8^{PA} (Panama CZ30.6, Marrero-1d)	1131	2224
T(VIL IR)T39M777 het-8^{PA}	7413	7412
het-8^{OR} (OR wild types)	2489	4200
chol-2 nit-6 het-8^{OR}	7212	--
ser-6 het-8^{OR} *ad-8*	--	7213
T(VIL→IR)T39M777 het-8^{OR}	2133	2134
T(VIL→IR)T39M777 nit-6 het-8^{OR}	7409	7408
T(VIL→IR)T39M777 ser-6 het-8^{OR}	7406	7407
T(VIL IR)T39M777 ad-8 het-8^{OR}	3187	3188

het-8^{HO} (Houma-1n, 1L)	2220	3943
chol-2 nit-6 ser-6 het-8^{HO}	7485	7486
T(VIL→IR)T39M777 het-8^{HO}	--	7411
het-9 (VIR)		
het-9^{PA} (Panama CZ30.6)	1131	2190
het-9^{OR} (OR wild types)	2489	4200
T(VIR→IVR)AR209 het-9^{OR}	1931	1932
het-10 (VIIR)		
het-10^{CR} (Costa Rica UFC205a)	851	--
het-10^{OR} (OR wild types)	2489	4200
T(VIIR→IL)5936 het-10^{OR}	2104	2105
mat (*mating type*) (IL)		
In *a^{m1}*, the mating and *het*-incompatibility functions of *mat a* are both inactive. In *a^{m33}, the het* function is inactive but the *mat a* mating function remains intact. *tol* is an unlinked recessive suppressor of *A/a het*-incompatibility.		
a^{m1} ad-3B cyh-1	--	(4564)
a^{m33}	--	5382
a^{m33} arg-3	--	5383
a^{m33} ad-3B	--	4568
tol (N83)	2338	1946
tol trp-4	2336	2337
leu-3 suc; tol pan-1	--	7322
leu-3 cyt-1 arg-3; tol	7337	--
T(IL→IIR)39311	1245	1246
T(IL→IIR)39311 am^{33}	--	6705
T(IL→IIR)39311; tol trp-4	2985	2976
T(IL→IIR)39311 ser-3 arg-1; tol	--	3220
In(IL→IR)H4250	1563	1564
In(IL→IR)H4250; tol	1947	2975
In(IL→IR)H4250 leu-3; tol	3253	3254