Genetic manipulation of *Aspergillus nidulans*: heterokaryons and diploids for dominance, complementation and haploidization analyses

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The haploid microbial eukaryote *Aspergillus nidulans* is a powerful genetic system, which allows analysis of a broad range of biological phenomena. In addition to conventional analysis of meiotic progeny in a single generation, parasexual analysis affords a rapid and convenient method for genetic analysis. We describe the construction of *A. nidulans* heterokaryons and diploids for use in genetic analysis to determine dominance and conduct complementation tests. We also describe the rapid mapping of mutations to chromosomes by haploidization of diploids carrying marked chromosomes. Balanced heterokaryons may be established within 10 days and diploids may be constructed in 2–3 weeks. Dominance tests and complementation tests using balanced heterokaryons or diploids may be completed in 2–3 days. Haploidization analysis of heterozygous diploids can be achieved within 10 days. These protocols should be adaptable for use in related Aspergilli and Penicillia, which lack a known meiotic cycle.

INTRODUCTION

The microbial eukaryote *A. nidulans* is an excellent genetic model system, and studies in this organism have contributed considerably to our understanding of many biological processes[1]. The sexual cycle of *A. nidulans* allows for conventional genetic analysis of meiotic progeny (**Fig. 1**) (see ref. 1). Genetic analysis of *A. nidulans,* which is normally haploid, may also be carried out, exploiting the parasexual cycle to form heterokaryons and diploids (**Fig. 1**). *A. nidulans* strains may fuse to form heterokaryons—hyphae containing nuclei of two different types. In a balanced heterokaryon derived from parents carrying balancing selectable markers, there will be approximately equal representation of nuclei from each parent[2]. Balanced heterokaryons may be used for complementation analysis and for dominance tests[3] (see ref. 4 for a detailed discussion), or to test for cytoplasmic inheritance[5,6]. Heterokaryons spontaneously formed during gene replacement of essential genes by DNA-mediated transformation may be used to propagate the deletion mutant nucleus, demonstrate the gene to be essential and determine the phenotype of the mutant allele (see refs. 7,8).

A. nidulans may form stable diploids by the occasional fusion of nuclei (less than 10^{-6}) within a heterokaryon (**Fig. 1**) (see refs. 2,3). Diploid strains can be grown in the same manner as haploid strains. *A. nidulans* diploids have a larger nuclear volume than haploids and diploid conidia are larger than haploid conidia[3,9]. Diploids may be used for dominance studies and selection of dominant mutations. During mitotic divisions, a diploid may lose one chromosome, giving rise to a $2n-1$ aneuploid[10]. Aneuploids are unstable, losing additional chromosomes until haploidy is reached. The process of generating haploid strains from a diploid is referred to as haploidization. The low spontaneous frequency with which *A. nidulans* diploids are destabilized may be increased by treatment with chemicals that interfere with the mitotic spindle, allowing efficient recovery of stable haploid segregants carrying a copy of each homologous chromosome[11,12]. As chromosomes are lost randomly from the diploid, it is possible to map a mutation to a chromosome (linkage group) or to assess whether a recessive mutation is haploid lethal by scoring the resulting haploids for the presence or absence of genetically marked chromosomes.

We describe protocols for establishing heterokaryons for dominance and complementation analysis, and construction of diploids for dominance studies and complementation tests and for haploidization analysis. These protocols should also be successful in related Aspergilli and Penicillia.

Dominance tests

Dominance tests are used to provide information about the nature of a mutation by assessing the phenotype of the mutant in the presence of a wild-type allele (**Fig. 2a**). Loss-of-function mutations are usually recessive to the wild type, whereas gain-of-function mutations are normally dominant or semi-dominant. Dominance tests require the mutant and wild-type alleles to be present in the same cell and therefore may be carried out in balanced heterokaryons carrying mutant and wild-type nuclei[3,13] or in heterozygous diploids[3]. It should be noted, however, that *A. nidulans* heterokaryons often are unbalanced, with varying relative numbers of each kind of nucleus leading to variable ratios of alleles. Therefore, it is recommended to use diploids, which carry one copy of the mutant allele and one copy of the wild-type allele per nucleus, for dominance tests.

Complementation tests

Complementation tests are used to determine whether two independently isolated recessive mutations affect the same gene function by introducing the mutations into the same cell[3,13–15]. In *A. nidulans,* mutations can be introduced to the same cell in separate nuclei in a heterokaryon or in the same nucleus in a heterozygous diploid (**Fig. 2b**). Heterokaryons formed between two mutants may be used to test whether two mutations affect the same gene product, that is, whether two mutations complement each other. Mutations in the same gene will not usually complement, whereas mutations in different genes complement each other because each nucleus provides the gene product missing from the other. Complementation tests may be performed in diploids by observing the phenotype of

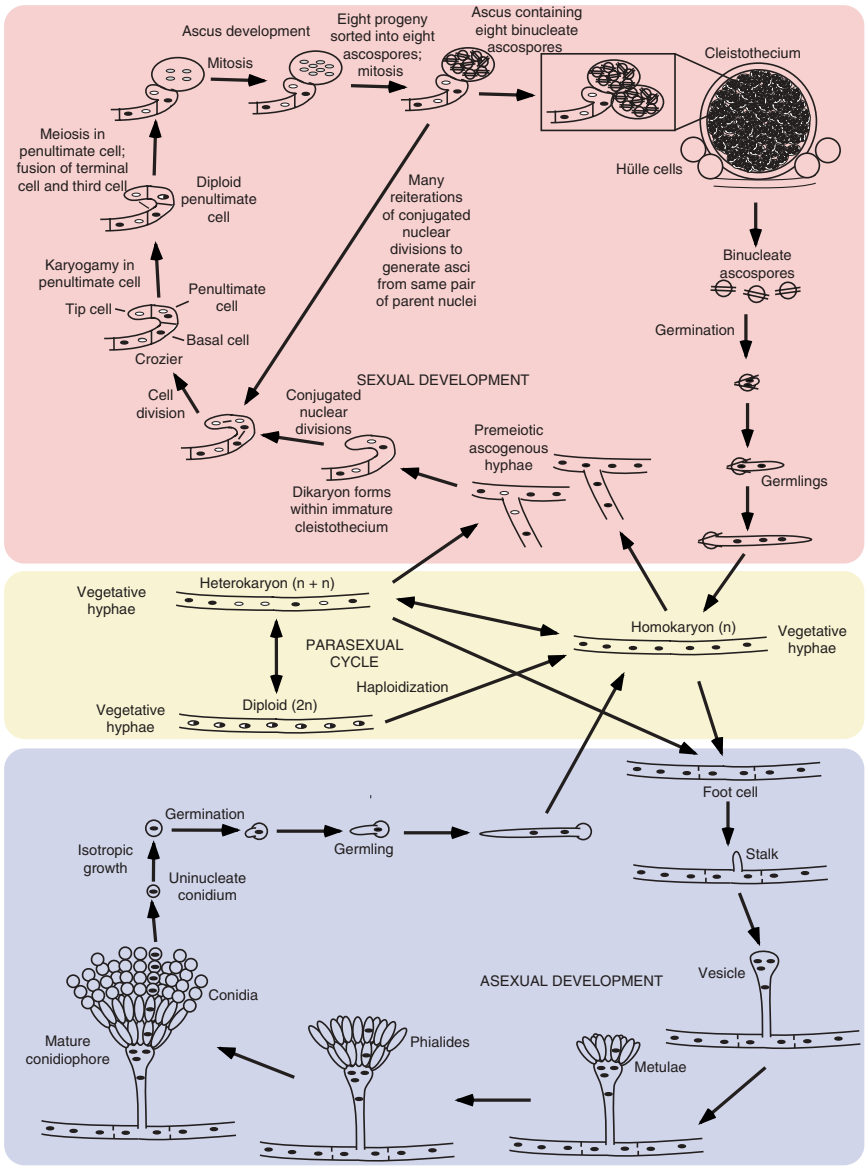


Figure 1 | Life cycle of *A. nidulans*. *A. nidulans* grows as haploid vegetative filamentous hyphae following germination of uninucleate conidia or binucleate ascospores. Vegetative hyphae from two individuals may fuse to form a heterokaryon, and nuclei in a heterokaryon or a homokaryon may fuse to form a diploid. Vegetative hyphae differentiate by asexual development to produce spores (conidia) on specific structures called conidiophores. Asexual development is initiated by differentiation of a specialized foot cell from which a stalk emerges. A vesicle is formed at the end of the stalk and two layers of uninucleate sterigmata—metulae and phialides—and uninucleate spores (conidia) are elaborated by successive budding from the vesicle. As growth proceeds, sexual development (highlighted in pink) occurs by the production of presumed nurse cells called Hülle cells and closed sexual fruiting bodies (cleistothecia) containing sexual spores (ascospores) arranged in nonlinear asci. Each cleistothecium contains as many as 10,000 ascospores that are the meiotic progeny of a single ascogenous hypha[3]. Asci arise from croziers, which are formed by simultaneous division of the two nuclei of the terminal cell of an ascogenous hypha[19]. The crozier comprises a uninucleate tip cell, a binucleate penultimate cell and a uninucleate basal cell. The ascus is formed by enlargement of the penultimate cell, which is accompanied by fusion of its two nuclei to form a transient diploid zygote that undergoes meiosis immediately. The four nuclei produced by meiosis divide mitotically to generate eight nuclei, each of which is included in one of the eight dark red ascospores in each ascus. The nucleus in each ascospore undergoes a single mitotic division resulting in mature binucleate ascospores. The tip and basal cells fuse to generate a binucleate cell, which forms a second crozier and the process is repeated. All of the ascospores within a single cleistothecium are of selfed or hybrid origin, which may arise if all of the dikaryotic ascogenous hyphae (and therefore all of the asci) are generated by conjugated divisions of one pair of parental nuclei[3]. The formation of heterokaryons and diploids, and haploidization of diploids described in this protocol rely on the parasexual cycle in the center of the figure (highlighted in yellow). Clonal propagation for growth testing and replica plating relies on the production of asexual conidia, shown in the lower part of the figure (highlighted in purple).

individuals heterozygous for the mutations under study. If a wild-type phenotype is observed, then the mutations complement each other and therefore are usually in different genes. If a mutant phenotype is observed, then the mutations do not complement each other and are in the same gene.

In rare cases, complementation between two mutations may be observed when the two mutations are in a diploid nucleus but not when they are in separate nuclei in heterokaryons[4,16]. Such an outcome may arise on account of unbalanced heterokaryons leading to the complementary nuclei being insufficiently close together for the complementary gene products to fruitfully combine. Alternatively, the gene products may be limited to their nucleus of origin.

Haploidization analysis of diploids

Haploidizataion of a diploid heterozygous for two markers *a* b^+/a^+ *b* gives two possible segregation patterns among the haploid progeny depending on whether *a* and *b* are on the same chromosome or different chromosomes (**Fig. 3**). When *a* and *b* are on the same chromosome, only two haploid genotypes are possible: *a* b^+ and a^+ *b*. If *a* and *b* are on different chromosomes, then all four combinations of haploid segregant are possible: *a* b^+, a^+ *b*, *a b* and a^+ b^+. The frequency of mitotic recombination is very low relative to whole chromosome loss[11].

Haploidization analysis of diploids formed between a mutant and a master strain marked for each linkage group allows an unmapped gene to be assigned to a linkage group. *A. nidulans* Master Strain F (MSF) is marked for each of the eight linkage groups (I: *yA2, adE20 suA(adE20)*; II *AcrA1*; III *galA1*; IV *pyroA4*; V *facA303*; VI *sB3*; VII *nicB8*; VIII *riboB2*)[11]. Diploids formed between a mutant and MSF can be used in haploidization analysis to assign mutations to linkage groups as the mutation will segregate independently of all the MSF markers except for one, which will show

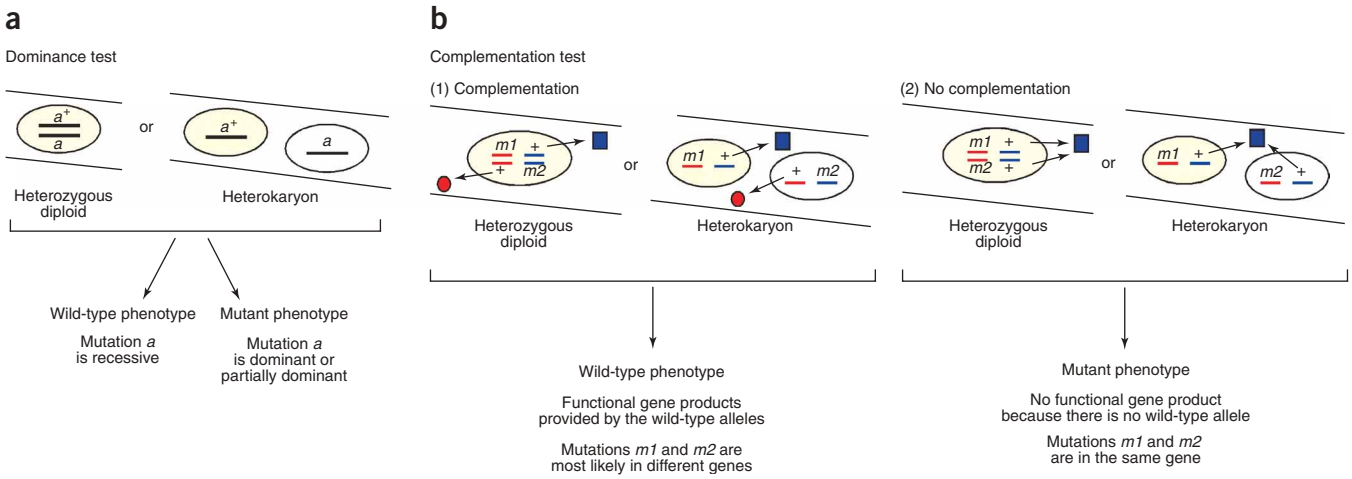


Figure 2 | Tests for dominance and complementation. (**a**) Dominance tests determine the function of a mutant allele (*a*) in the presence of a wild type allele (a^+) in heterozygous diploids or in heterokaryons. If a wild-type phenotype is observed, the mutation is recessive. If a mutant phenotype is observed, the mutation is partially or completely dominant. (**b**) Complementation tests determine whether two recessive mutations are in the same gene or in different genes. The two mutations *m1* and *m2* are brought together in a heterozygous diploid or in a heterokaryon. (1) Complementation. A wild-type phenotype is observed if complementation occurs. The wild-type allele of each gene affected by mutation provides a functional gene product. The mutations are complementary and are most likely in different genes. (2) No complementation. A mutant phenotype is observed if the mutations do not complement each other. No functional gene product is produced as the mutations are in the same gene and both alleles of the affected gene are mutated.

segregation in repulsion to the mutation (**Fig. 4**). The mutation therefore lies on the chromosome homologous to the chromosome carrying this particular marker.

Haploidization may be used to detect translocations[17,18]. If a diploid is constructed between a marked master strain and a strain carrying a reciprocal translocation between two chromosomes, the linkage relationships between the markers will be altered in the haploid segregants (**Fig. 5**). Usually, the haploids containing duplications and deficiencies are not viable and therefore are not recovered, and the only haploids recovered are the balanced translocation and normal haploids. The markers on the two chromosomes involved in the translocations will always segregate together in the parental combinations in the haploids.

MATERIALS

REAGENTS

- Strains: *A. nidulans* strains are available from the Fungal Genetics Stock Center (http://www.fgsc.net/) ▲ CRITICAL Chemicals may be purchased from standard chemical supply companies, except where indicated.
- Sodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$)
- Cupric sulfate ($CuSO_4 \cdot 5H_2O$)
- Ferric orthophosphate ($FePO_4 \cdot 4H_2O$)
- Manganous sulfate ($MnSO_4 \cdot H_2O$)
- Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)
- Zinc sulfate ($ZnSO_4 \cdot 7H_2O$)
- Potassium chloride (KCl)
- Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)
- Potassium dihydrogen phosphate (KH_2PO_4)
- *p*-Amino benzoic acid
- Aneurin (thiamine · HCl)
- Biotin
- Nicotinic acid
- Calcium D-pantothenate
- Pyridoxine · HCl
- Riboflavin
- Casein hydrolysate
- D-Glucose
- Ammonium tartrate
- Peptone
- Yeast extract
- Agar
- Sodium nitrate

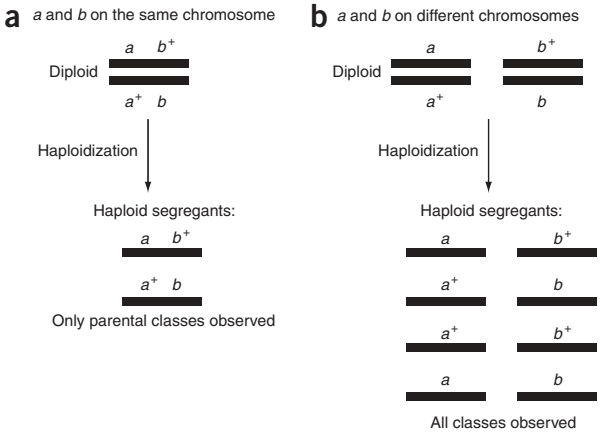
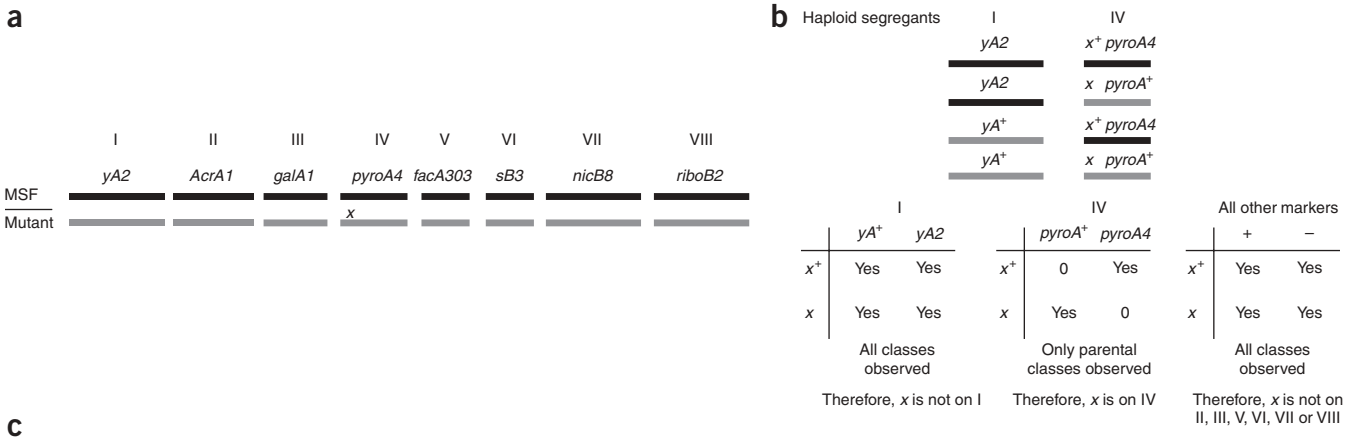


Figure 3 | Segregation of markers during haploidization of *A. nidulans* heterozygous diploids. Haploids are generated by haploidization of a diploid on complete medium containing benlate and are then genotyped on test media as described in **Box 3**. (**a**) Markers on the same chromosome segregate in repulsion as whole chromosomes segregate together giving only the parental combinations of markers and no recombinants among haploid progeny. (**b**) Markers on different chromosomes segregate independently generating all four possible combinations of markers among the haploid progeny.



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Test media:	Complete	Complete acriflavine	Carbon-free NH_4 Galactose	Carbon-free NH_4 Acetate	ANM NO_3 Biot Pyro Nic Thio Ribo	ANM NH_4 Biot Pyro Nic Thio Ribo	ANM NH_4 Biot Pyro Nic Ribo	ANM NH_4 Biot Pyro Thio Ribo	ANM NH_4 Biot Nic Thio Ribo	ANM NH_4 Pyro Nic Thio Ribo	ANM NH_4 Biot Pyro Nic Thio	
Marker:	N/A	*AcrA1*	*galA1*	*facA303*	*niiA4*	N/A	*sB3*	*nicB8*	*pyroA4*	*biA1*	*riboB2*	Inferred
Chromosome:	N/A	II	III	V	VIII	N/A	VI	VII	IV	I	VIII	genotype
Colony number: 1	+	–	+	+	+	+	+	+	+	+	+	WT
2	+	–	+	+	–	+	+	+	+	+	+	*niiA4*
3	+	+	+	+	+	+	+	+	+	+	+	*AcrA1*
4	+	–	–	+	+	+	+	+	+	+	+	*galA1*
5	+	–	+	+	+	+	+	+	–	+	+	*pyroA4*
6	+	–	+	+	+	+	+	+	+	–	+	*biA1*
7	+	–	+	+	+	+	+	–	+	+	+	*nicB8*
8	+	–	+	+	+	+	–	+	+	+	+	*sB3*
9	+	–	+	–	+	+	+	+	+	+	+	*facA303*
10	+	–	+	+	+	+	+	+	+	+	–	*riboB2*
11	+	–	+	+	+	+	+	–	+	–	+	*nicB8 biA1*
12	+	+	–	+	+	+	+	+	+	+	+	*AcrA1 galA1*
13	+	–	+	–	–	+	+	+	–	+	+	*pyroA4 facA303 niiA4*
MSF	+	+	–	–	+	–	–	–	–	+	–	*AcrA1 galA1 pyroA4 facA303 sB3 nicB8 riboB2*
MH54	+	–	+	+	–	+	+	+	–	+	+	*biA1 niiA4*

Figure 4 | Assignment of a mutation to a chromosome by haploidization of an *A. nidulans* heterozygous diploid. (**a**) The genotype of a diploid constructed between a mutant (carrying a mutation *x* on chromosome IV) and MSF (carrying a marker on each chromosome). (**b**) All possible combinations of haploid segregants for chromosomes I and IV following haploidization on complete medium containing benlate are shown. (**c**) Genotype determination by growth tests. The genotype of *A. nidulans* strains may be inferred from growth tests on test media designed to score a particular marker. Sample growth test data and inferred genotypes for 13 haploid colonies and the parents of the diploid in the example (MSF and MH54) are shown. The relevant components of the test media and the growth (+) or poor or no growth (–) of the strains are shown. The marker and marked chromosome scored on each medium are indicated. ANM (*Aspergillus* Nitrogen–free medium), Biot (Biotin), Pyro (pyridoxine), Nic (nicotinic acid), Thio (thiosulfate), Ribo (riboflavin), N/A, not applicable. Conidial color (not shown) is scored by visual inspection on complete medium as green (wild type) or yellow (*yA2*). The *yA2* marker is on chromosome 1.

- Sodium hydroxide
- Ammonium chloride
- D-Galactose
- Acriflavine
- Nicotinic acid
- Thiosulfate
- Sodium acetate
- Benlate fungicide (Du Pont)
- Trace elements solution (see REAGENT SETUP)
- Salts solution (see REAGENT SETUP)
- Vitamin solution (see REAGENT SETUP)
- Complete medium (see REAGENT SETUP)
- *Aspergillus* nitrogen-free medium (ANM) (see REAGENT SETUP)
- Nitrogen sources, for example, ammonium tartrate, sodium nitrate (see REAGENT SETUP)
- Supplements; riboflavin, biotin, pyridoxine, nicotinic acid and thiosulfate (see REAGENT SETUP)

EQUIPMENT

- Growth chambers (incubators) at 37 °C
- Double-wire twist replicator
- 26-prong double wire twist replicator
- Plastic Petri plates (90 mm)
- Bunsen burner
- Dissecting microscope

REAGENT SETUP

▲ **CRITICAL** All solutions and media are made up in deionized water.

Trace elements solution (per liter) 0.04 g sodium tetraborate, 0.4 g cupric sulfate, 1.0 g ferric orthophosphate, 0.6 g manganous sulfate, 0.8 g sodium molybdate, 8.0 g zinc sulfate. Add 2.0 ml chloroform as a preservative. Store at 4 °C.

Salts solution (per liter) 26.0 g KCl, 26.0 g $MgSO_4 \cdot 7H_2O$, 76.0 g KH_2PO_4, 50 ml trace elements solution; pH should be approximately 4.0. Add 2.0 ml chloroform as a preservative. Store at 4 °C.

Vitamin solution (per liter) 20.0 mg *p*-amino benzoic acid, 50.0 mg aneurin (thiamine · HCl), 1.0 mg biotin, 100 mg nicotinic acid, 200 mg calcium D-pantothenate (pantothenic acid calcium salt), 50 mg pyridoxine · HCl, 100 mg riboflavin. Store wrapped in foil (riboflavin is light-sensitive) at 4 °C.

Complete medium (per liter) 20 ml salts solution, 10 ml vitamin solution, 10 ml of 150 g per liter casein hydrolysate, 10.0 g D-glucose, 61.84 g ammonium tartrate, 2.0 g peptone, 1.0 g yeast extract. Adjust to pH 6.5. Add 1.0% or 2.2% (w/v) agar. Autoclave. Solid complete media may be conveniently stored in bottles at room temperature (18–25 °C).

Preparation of complete media plates Melt the complete media using a microwave oven. Allow the media to cool to approximately 56 °C (it may be prudent to place the media in a 56 °C water bath to prevent it from setting). Pour approximately 20–25 ml complete media into 90 mm plastic Petri plates. Allow the media to set. Plates may be poured weeks before use and stored at 4 °C. ! CAUTION Ensure that bottle caps are loosened before microwaving the media so that bottles do not explode. ▲ CRITICAL Before inoculation, dry the plates on the bench overnight or in a laminar flow hood for 30 min. This reduces spore scatter.

***Aspergillus* Nitrogen–free medium (ANM) (per liter)** 20.0 ml salts solution, 10.0 g D-glucose. Adjust to pH 6.5. Add 1.0% (w/v) agar. Autoclave. Solid ANM may be conveniently stored in bottles at room temperature (18–25 °C).

Preparation of ANM plates Melt the ANM using a microwave oven. Allow the media to cool to approximately 56 °C (it may be prudent to place the media in a 56 °C water bath to prevent it from setting). Add the desired nitrogen source and required supplements (see below). Gently mix. Pour approximately 20–25 ml ANM into 90 mm plastic Petri plates. Allow the media to set. ! CAUTION Ensure that bottle caps are loosened before microwaving the media so that bottles do not explode. ▲ CRITICAL Before inoculation, dry the plates on the bench overnight or in a laminar flow hood for 30 min. This reduces spore scatter.

Nitrogen sources Prepare nitrogen sources as 1.0 M solutions. Autoclave. Add nitrogen sources at 10 mM (1.0 ml of 1.0 M solution per 100 ml minimal medium) to autoclaved carbon-free or nitrogen-free medium. Ammonium tartrate ($(NH_4)_2T$) or sodium nitrate (Na_2NO_3) are nitrogen sources that support strong growth.

***Aspergillus* carbon-free medium (per liter)** 20.0 ml salts solution. Adjust to pH 6.5. Add 1.0% (w/v) agar. Autoclave. Solid carbon-free media may be stored in bottles at room temperature. For growth testing, carbon sources are added at the desired concentration to *Aspergillus* carbon-free medium. The plates are poured as for ANM, including the addition of a nitrogen source and required supplements.

Galactose medium D-Galactose may be prepared as a 10.0% (w/v) solution. Add 1.0% (w/v) D-galactose to *Aspergillus* carbon-free medium (for scoring *galA1* mutants, which are unable to use galactose as a carbon source). ▲ CRITICAL A nitrogen source (e.g., 10 mM ammonium chloride) must also be added. Use ammonium chloride rather than ammonium tartrate as a nitrogen source when testing carbon source utilization, as tartrate is a carbon source and may make scoring difficult.

Acetate medium Prepare a solution of 1.0 M sodium acetate pH 6.5. Add 50 mM sodium acetate pH 6.5 to *Aspergillus* carbon-free medium (for scoring *facA303*, which is defective in acetate utilization). ▲ CRITICAL A nitrogen source (e.g., 10 mM ammonium chloride) must also be added. Use ammonium chloride rather than ammonium tartrate as a nitrogen source when testing carbon source utilization, as tartrate is a carbon source and may make scoring difficult.

Acriflavine Add 100 μg ml^{-1} acriflavine to molten complete medium (for scoring *AcrA1* mutants, which are resistant to acriflavine) and mix well before pouring plates. ! CAUTION Wear gloves, as acriflavine is toxic.

Benlate fungicide Prepare benlate stock solution at 0.1 mg ml^{-1} in 100% methanol. Prepare 1 μg ml^{-1} benlate media: add 1 ml of 0.1 mg ml^{-1} benlate per 100 ml complete media after autoclaving. ▲ CRITICAL To ensure that benlate goes into solution, add benlate stock solution to complete media while the medium is still hot.

Riboflavin Prepare a 250 μg ml^{-1} riboflavin working stock. Autoclave. Store wrapped in a foil (as riboflavin is light-sensitive). Add 1.0 ml per 100 ml minimal medium as required (to support growth of strains carrying the *riboB2* mutation).

Biotin Prepare a 1.0 μg ml^{-1} biotin working stock. Autoclave. Add 1.0 ml per 100 ml minimal medium as required (to support growth of strains carrying the *biA1* mutation).

Pyridoxine Prepare a 50 μg ml^{-1} pyridoxine·HCl working stock. Autoclave. Add 1.0 ml per 100 ml minimal medium as required (to support growth of strains carrying the *pyroA4* mutation).

Nicotinic acid Prepare a 0.1 mg ml^{-1} nicotinic acid stock. Autoclave. Add 1.0 ml per 100 ml minimal medium as required (to support growth of strains carrying the *nicB8* mutation).

Thiosulfate Prepare a 1.0 M thiosulfate stock. Autoclave. Add 1.0 ml per 100 ml minimal medium as required (to support growth of strains carrying the *sB3* mutation).

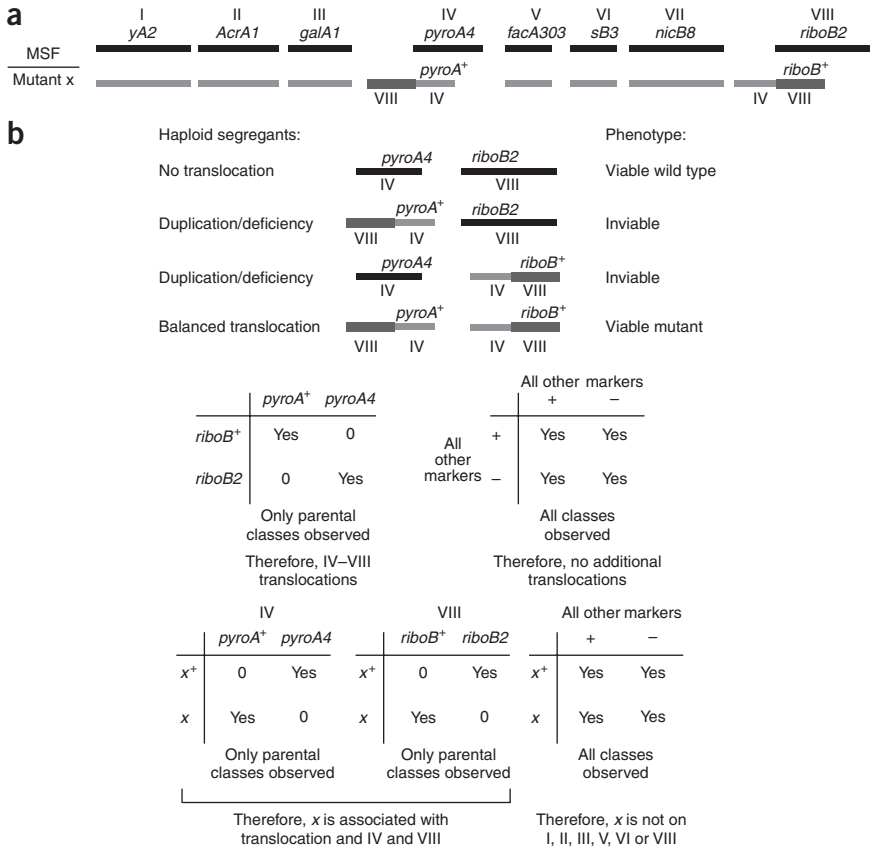

Figure 5 | Assignment of a reciprocal translocation to chromosomes by haploidization of an *A. nidulans* heterozygous diploid. (**a**) The genotype of a diploid constructed between a mutant (carrying a mutation *x* associated with a reciprocal translocation between chromosomes IV and VIII) and MSF (carrying a marker on each chromosome). The IV–VIII translocated chromosome carries the *riboB*$^+$ allele and the VIII–IV translocated chromosome carries the *pyroA*$^+$ allele. (**b**) All possible combinations of haploid segregants for chromosomes IV and VIII following haploidization on complete medium containing benlate are shown. The translocation is detectable by the association of chromosome IV and chromosome VIII markers in the parental combination and the segregation of the mutant phenotype in repulsion with chromosome IV and chromosome VIII markers. For translocations that lack a mutant phenotype, the recovery of only the parental combination of certain MSF markers indicates the chromosomes involved in the translocation.

PROCEDURE

Heterokaryon formation ● TIMING 6–10 days

1| Stab-inoculate the two parent strains 5 mm apart on solid complete medium.

▲ CRITICAL STEP The parent strains must be inoculated close to each other for anastomoses (hyphal fusions) to occur. Colonies inoculated further apart than 5 mm may show contact inhibition and the parents may not meet.
▲ CRITICAL STEP Use parent strains with complementary nutritional requirements to force the heterokaryon. The complementary nutritional requirements select for the maintenance of a heterokaryon with a balance of each type of parental nucleus.
▲ CRITICAL STEP It is valuable to use parents carrying different conidial color markers—this allows heterokaryons to be easily identified by the production of conidia of different colors at Step 5. In the example, we have used a yellow (*yA2*) mutant and a wild-type (green) conidial color strain (**Fig. 6**).

In heterokaryons constructed between a *yA2* mutant and a white (*wA3*) mutant, diploid conidia will be obvious due to their green (wild type) color produced because of complementation of the conidial color mutations.
■ PAUSE POINT *A. nidulans* is very tolerant to pauses in experimental procedures. Plates may be left at room temperature for several days or stored for longer periods (up to several months) at 4 °C. Growth will resume upon return to 37 °C.

2| Incubate for 2 days at 37 °C (**Fig. 6a**).
? TROUBLESHOOTING

3| Using a sterile wire twist, cut out the non-conidiating edge of the colony at the point of fusion between the two strains (**Fig. 6b**). Transfer the agar plug to a plate containing solid selective medium selecting for the heterokaryon or diploid and selecting against both parents (**Fig. 6c**).

In the example (**Fig. 6**), strains MH54 (*biA1 niiA4*) and MSF (*yA2 adE20 suA(adE20) AcrA1 galA1 pyroA4 facA303 sB3 nicB8 riboB2*) are transferred to selective medium (ANM plus nitrate lacking supplements). The MH54 parent is selected against because of the lack of biotin in the medium and inability to use nitrate as a nitrogen source and MSF is selected against because of the lack of pyridoxine, nicotinic acid and riboflavin in the medium. An MH54 + MSF heterokaryon will be able to synthesize all the nutritional requirements for growth.

4| Incubate at 37 °C for 2 days.

5| Repeat transfer of the colony edge to fresh plates of the same selective medium and incubate at 37 °C for 3 days until a balanced heterokaryon characterized by strong growth and an equal mixture of conidial colors is established (**Fig. 6d–f**). Usually a minimum of two repeats of the transfer is required.
? TROUBLESHOOTING

6| To use the heterokaryons for dominance tests or complementation analysis, proceed with growth tests as detailed in **Box 1**. To form diploids for subsequent dominance tests, complementation analysis or haploidization analysis, proceed directly to Step 7.

Diploid construction ● TIMING **2–3 weeks**

7| Prepare a dilute conidial suspension from the heterokaryon (Step 5; **Fig. 6f**) by scraping a loop-full of conidia and suspending in 0.4 ml of 0.005% Tween 80. Note, 0.005% Tween 80 is used to avoid clumping of hydrophobic conidia.

8| Spread approximately 100 µl conidial suspension per plate on selective media (**Fig. 6g**). Alternatively, 400 µl conidial suspension may be added to 100 ml molten media (cooled to less than 55 °C), mixed well and used for four plates.

9| Incubate for 2–3 days at 37 °C. Diploid conidia will form colonies on selective medium. Haploid conidia will not form single colonies on selective media as *A. nidulans* conidia are uninucleate.

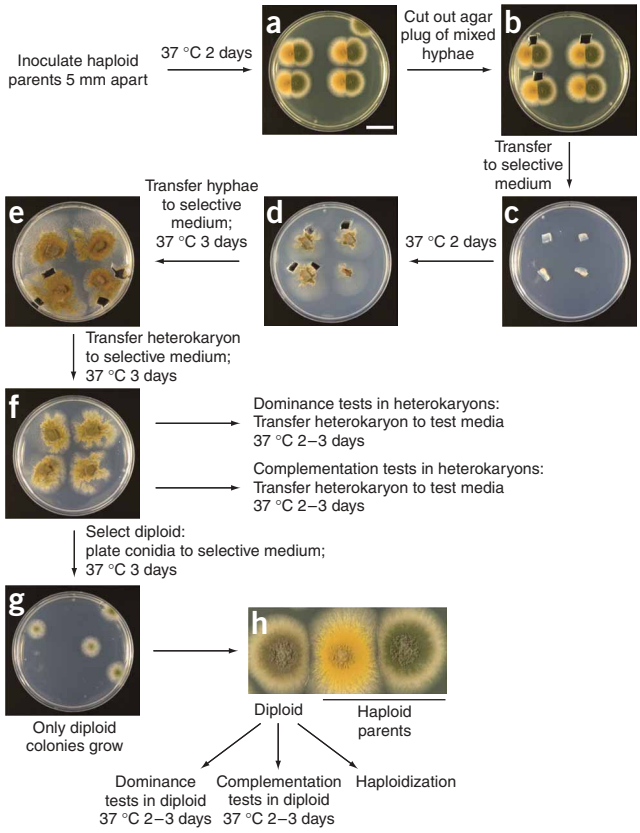

Figure 6 | Construction of *A. nidulans* heterokaryons and diploids. (**a**) The two parent strains, MH54 (*biA1 niiA4*) and MSF (*yA2 adE20 suA(adE20) AcrA1 galA1 pyroA4 facA303 sB3 nicB8 riboB2*), are inoculated 5 mm apart on a 1.0% complete medium plate and incubated for 2 days at 37 °C. Scale bar, 20 mm. (**b**) An agar plug of mixed hyphae is cut out and transferred to selective medium (**c**) and incubated at 37 °C for 2 days. (**d**) An agar plug of hyphae is cut out and transferred to selective medium and incubated at 37 °C for 3 days to form a heterokaryon (**e**). The heterokaryon is cut out of the agar and transferred to selective medium and incubated at 37 °C for 3 days (**f**). The balanced heterokaryon may be used for dominance tests, complementation tests or to select diploids. Diploids are selected by plating conidia to selective medium and incubating at 37 °C for 3 days (**g**); only diploid colonies will be able to grow. The pale green diploid is shown with the dark green (wild type) and *yA* (yellow) mutant parents (**h**). Diploids may be used for dominance tests, complementation tests or mapping a mutation to a chromosome by haploidization analysis.

BOX 1 | GROWTH TESTS OF HETEROKARYONS FOR DOMINANCE TESTS AND COMPLEMENTATION TESTS ● TIMING 2–3 DAYS

The conidia and heterokaryons of *A. nidulans* can be readily transferred on wire twists to solid media. The relative growth of the discrete compact colonies can then be assessed according to colony diameter, mycelial density and the degree of conidiation. For a detailed description of *A. nidulans* growth testing, see Todd *et al.*[1]

1. Transfer the heterokaryon to the test media by cutting out a small agar plug of heterokaryotic hyphae and physically transfer the plug.
▲ CRITICAL STEP Growth tests of heterokaryons cannot be performed by conidial transfer, as *A. nidulans* conidia are uninucleate.
▲ CRITICAL STEP Transfer homokaryotic hyphal plugs of the parents of the heterokaryon and a wild-type control for comparison.
2. Incubate for 2–3 days at 37 °C.
3. Score the growth of the heterokaryon compared with the controls. For expected outcome, see ANTICIPATED RESULTS.

In the example (**Fig. 6**) using strains MH54 (*biA1 niiA4*) and MSF (*yA2 adE20 suA(adE20) AcrA1 galA1 pyroA4 facA303 sB3 nicB8 riboB2*), the selective medium is ANM plus nitrate lacking supplements. An MH54/MSF diploid will be able to synthesize all the nutritional requirements for growth due to complementation. The MH54 parent is selected against due to inability to utilize nitrate and the lack of biotin in the medium and MSF is selected against due to the lack of pyridoxine, nicotinic acid and riboflavin in the medium.

▲ CRITICAL STEP Diploid colonies formed between a *yA2* mutant and a wild-type conidial color strain produce pale green conidia (**Fig. 4h**). Diploid colonies formed between strains with different conidial color mutations will produce a conidial color phenotype dependent on their genotype.

▲ CRITICAL STEP Diploid colonies may break down at low frequency. Maintain diploids by continued growth and storage on selective media.

? TROUBLESHOOTING

10| To determine the dominance of a particular mutation or complementation of two recessive mutations, analyze diploid colonies using growth tests detailed in **Box 2**. To map a mutation to a chromosome using diploids constructed between a mutant and MSF, proceed directly to Step 11 for haploidization analysis.

Haploidization analysis ● TIMING Should be complete within 10 days

11| Stab-inoculate conidia of the required diploid strain onto plates containing complete medium and 1 μg ml^{-1} benlate. Incubate for 5–7 days at 37 °C.

12| Haploids derived from a $yA^+/yA2$ diploid as in the example will be apparent as small dark green or yellow sectors (**Fig. 7**). There will be pale green diploid sectors, which will be identified later as being wild type in phenotype for all recessive markers.

▲ CRITICAL STEP The frequency of haploidization can be increased by the use of *p*-fluorophenylalanine[11] or 1 μg ml^{-1} benlate fungicide[12], which acts to disrupt mitosis.

? TROUBLESHOOTING

13| Pick haploid sectors onto complete medium. Incubate for 2 days at 37 °C. Note that haploid colonies derived from diploids containing different combinations of conidial color mutations will show a conidial color phenotype dependent on their genotype and this may differ from the example yA^+/MSF diploid described in **Figure 6**.

14| Purify by streaking for single colonies on complete media. Incubate for 2 days at 37 °C.

15| Pick haploid segregants to set up a master plate (see **Box 3**). Incubate for 2 days at 37 °C.

16| Replica plate for growth testing (see **Box 3**). Include wild type and both haploid parents as controls.

BOX 2 | GROWTH TESTS OF DIPLOIDS FOR DOMINANCE TESTS AND COMPLEMENTATION TESTS ● TIMING 2–3 DAYS

Diploid strains may be growth tested in the same manner as haploid strains by conidial transfer on sterile wire twists.

1. Stab-inoculate the diploid to the test media.
▲ CRITICAL STEP Include the parents of the diploid and a wild-type strain as controls of known genotype.
2. Incubate at 37 °C for 2–3 days.
3. Score the growth of the diploid compared with the controls. For expected outcome, see ANTICIPATED RESULTS.

● TIMING

Dominance tests: once a diploid or balanced heterokaryon is established, dominance tests should be completed in 2–3 days.
Complementation analysis: once a diploid or balanced heterokaryon is established, complementation tests should be completed in 2–3 days.
Haploidization analysis: once a diploid has been formed, haploidization analysis should be completed within 10 days. Haploid segregants should be recovered in 5 days. Master plates grown in 2 days and replicated selective media plates require 2–3 days growth.

Figure 7 | Haploidization of *A. nidulans* diploids. Haploid segregants are recovered from diploids following repeated stab-inoculation on to complete medium containing 1 μg ml^{-1} benlate and incubation at 37 °C for 5 days. Yellow (*yA*) or dark-green haploid segregants are visible as sectors arising from the pale-green diploid parent. These are purified on complete medium for subsequent genotyping as described (**Box 3**; **Fig. 4c**). Scale bar, 5 mm (**a**) and 1 mm (**b**).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Solution
1–16 **Boxes 1–3**	Contamination	Ensure media is sterile; work using Bunsen burner
2	Strains have not grown	(a) Check media is of pH 6.5 (b) Check genotype of strains/requirements are added to media (c) Check that a nitrogen source was added to the media (d) The culture(s) used for inoculation may be dead. Use a fresh source of conidia for inoculation
2	The parent strains have not grown together	Inoculate the parents closer together in Step 1. Note that poorly growing mutants may need to be inoculated very close together
5	A strongly growing balanced heterokaryon has not formed after repeated transfer	Some nutritional markers are leaky and do not provide strong selection against the parent on selective medium (e.g., *biA1*). Use additional markers to provide strong selection against the parents
9	Haploid conidia of mixed genotype may clump together and form heterokaryotic colonies	Heterokaryotic colonies may be distinguished from diploid colonies by mixed conidial colors and by streaking for single colonies on selective media. Diploid conidia but not haploid conidia will give rise to single colonies
12	Haploid sectors have not formed. The diploid is severely inhibited in growth	Benlate is toxic. The concentration of benlate may be too high. Decrease the concentration of benlate in the media
12	Haploid sectors have not formed. The diploid shows no growth inhibition	The concentration of benlate may be too low. Increase the concentration of benlate in the media

BOX 3 | GROWTH TESTS OF A LARGE NUMBER OF STRAINS (e.g., FOLLOWING HAPLOIDIZATION) BY REPLICATING COLONIES TO APPROPRIATE TEST MEDIA FROM A MASTER PLATE ● TIMING 4–5 DAYS

1. Set up a master plate containing 26 colonies arranged in a grid on complete media.
2. Incubate for 2 days at 37 °C.
▲ CRITICAL STEP Use a template to mark out the spacing of the colonies to ensure accurate colony replication.
▲ CRITICAL STEP It can be extremely useful to include controls of known genotypes for comparison, including the parents.
3. Replicate the master plates to test media using a replicator with 26 nichrome wire twists (1.25 cm apart) set in a perspex base (see Todd *et al.*[1]). The wire twists are arranged in the pattern of the master plate grid.
4. Incubate the replica plates at 37 °C. The plates are scored after 2 or 3 days incubation and genotypes inferred (**Fig. 4c**). For expected outcome, see ANTICIPATED RESULTS.

ANTICIPATED RESULTS

Dominance tests

For dominance tests using heterokaryons or diploids, it is expected that either a wild-type phenotype or a mutant phenotype is observed (**Fig. 2a**). A wild-type phenotype indicates that the mutation in question is recessive to the wild type. A phenotype identical to the mutant parent indicates that the mutation of interest is dominant to the wild type. A mutant phenotype that is not identical to the mutant parent indicates that the mutation is partially dominant.

Complementation analysis

It may be expected for complementation tests of two recessive mutations in diploids or in heterokaryons that either a wild-type phenotype or a mutant phenotype will be observed (**Fig. 2b**). A mutant phenotype indicates a lack of complementation between the mutants and allows the conclusion that the mutations are in the same gene. A wild-type phenotype (complementation) indicates that the two mutations are most likely in separate genes. It should be noted that this latter conclusion is not absolute, as in rare cases particular mutations in a single gene may lead to intragenic complementation.

Haploidization analysis

In the haploid segregants, the unmapped gene will be linked in repulsion to one of the mutant alleles of the master strain because during the formation of haploid segregants whole chromosomes segregate together. As the master strain is marked for each chromosome, the mutation maps to the linkage group carrying the linked marker. The unmapped gene will segregate independently of all other marked chromosomes except if the mutant strain carries a translocation resulting in the mutant phenotype (**Fig. 4**). Translocations are detectable by cosegregation of the mutant gene in repulsion with two master strain markers and/or by cosegregation of two master strain markers (**Fig. 5**). Duplication or deficiency haploids are usually of low viability and therefore their presence is indicated by an under-represented (or more often absent) class.

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