Genetic manipulation of *Aspergillus nidulans*: meiotic progeny for genetic analysis and strain construction

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The multicellular microbial eukaryote *Aspergillus nidulans* is an excellent model for the study of a wide array of biological processes. Studies in this system contribute significantly to understanding fundamental biological principles and are relevant for biotechnology and industrial applications, as well as human, animal and plant fungal pathogenesis. *A. nidulans* is easily manipulated using classical and molecular genetics. Here, we describe the storage and handling of *A. nidulans* and procedures for genetic crossing, progeny analysis and growth testing. These procedures are used for Mendelian analysis of segregation of alleles to show whether a mutant phenotype segregates as a single gene and independent assortment of genes to determine the linkage relationship between genes. Meiotic crossing is used for construction of multiple mutant strains for genetic analysis. Genetic crossing and analysis of progeny can be undertaken in 2–3 weeks and growth testing takes 2–3 days.

INTRODUCTION

A. nidulans as a model organism

A. nidulans is an exceptional genetic model; indeed its power was recognized in an extensive screen by Pontecorvo *et al.*[1] for organisms with attributes ideal for genetic models. *A. nidulans* is a long-standing laboratory organism of high value for research and for teaching genetic principles and *A. nidulans* research has contributed much to our knowledge of a range of biological processes, including fundamental discoveries in the 1950s on gene action and recombination[2,3]. *A. nidulans* research has made major contributions to the understanding of spore development[4–7], cell cycle[8–12], cell polarity[13], DNA repair[14], metabolism and its control[15], secondary metabolism[16], signaling[17] and pH control[18,19].

The Aspergilli are filamentous ascomycetes and obligate aerobes. The metabolism and development of the model ascomycete yeast *Saccharomyces cerevisiae* is not representative of the Aspergilli and other filamentous ascomycetes. *S. cerevisiae* is a unicellular organism capable of aerobic and anaerobic growth. It divides by budding and does not produce asexual spores. *A. nidulans* is a representative model filamentous fungus, as it is closely related phylogenetically to other filamentous ascomycetes and shares a common lifestyle and growth forms. Moreover, *A. nidulans* has a well-defined sexual cycle, permitting genetic analysis, unlike many filamentous fungi, which lack a known sexual cycle, including the human pathogen *A. fumigatus*, the animal pathogen *A. flavus* and the devastating plant pathogen *A. parasiticus* and beneficial industrial fungi such as *A. oryzae*, *A. niger*, *A. terreus* and *Penicillium* spp used in food, enzyme, metabolite and antibiotic production. Also, along with *Neurospora crassa,* studies of *A. nidulans* are more broadly applicable to a wide range of other less closely related ascomycete pathogens, for example, Magnaporthe, Fusarium[20,21].

A. nidulans genome and useful resources

A. nidulans has eight linkage groups (n=8) and has a detailed genetic map[22]. Its genome is relatively small—30 Mb—and contains few repetitive elements. The *A. nidulans* genome sequence is publicly available via the Broad Institute (http://www.broad.mit.edu/annotation/fgi/)[23] along with the genome sequence of several other Aspergilli[24–26]. Useful resources for *A. nidulans* research include the following: Fungal Genetics Stock Center (http://www.fgsc.net/), Central Aspergillus Data Repository (http://www.cadre.man.ac.uk/), genetic maps, locus lists and nomenclature (http://www.gla.ac.uk/Acad/IBLS/molgen/aspergillus/) and a comprehensive book on *Aspergillus* research "*Aspergillus*: 50 years on"[27]. **Box 1** contains information about *Aspergillus* nomenclature and a glossary of terms.

Using *A. nidulans* for classical genetic analyses

A. nidulans has many advantages for genetic analysis, as listed below.

- An enormous metabolic versatility providing a range of systems for investigating catabolism and biosynthesis.
- Vegetative (hyphal) growth and two developmental programs—asexual development and sexual development (**Fig. 1**).
- Rapid growth to generate large amounts of biomass, thereby allowing growth testing of strains in several days.
- Liquid culture permits growth of mycelia for DNA preparation and transfer of mycelia to different growth conditions before RNA or protein extraction, thereby allowing studies of responses to environmental transitions.
- Of particular value is the compact colonial growth morphology on solid media, allowing the analysis of many individual colonies on a single Petri dish. Colonial growth may be assessed according to hyphal extension (colony diameter), hyphal density and the degree of asexual spore production (conidiation). The effects of media composition or mutations on growth rate or growth inhibition are readily distinguished.
- Availability of a collection of auxotrophic mutants for use in genetic analysis (see **Box 2**).
- An immense advantage is the production of uninucleate conidia, allowing clonal propagation and rapid purification of mutants, transformants and progeny from crosses.
- Sexual reproduction using a homothallic mating strategy, allowing both selfing and outcrossing[1]. The homothallic mating system obviates the need for generating mutants in the appropriate mating-type background for crossing, simplifying strain construction for genetic analysis.

BOX 1 | *A. nidulans* NOMENCLATURE

A. nidulans has two names. The name *A. nidulans* describes the asexual morph whereas *Emericella nidulans* describes the sexual form. There is a general consensus within the *Aspergillus* research community to use the name "*Aspergillus*" rather than "*Emericella*" irrespective of whether the asexual or sexual form is being described. Consequently, "*Aspergillus*" is the widely accepted name in the literature and genome annotation databases. However, it should be noted that certain GenBank/NCBI entries use "*Emericella*."

A. nidulans genes are normally referred to as a three-letter lowercase gene name followed by a capital letter locus designation (http://www.gla.ac.uk/Acad/IBLS/molgen/aspergillus/).

Glossary

Anastomoses: hyphal fusions
Ascospores: sexual spores
Conidia: asexual spores, which bud from the phialides
Conidiation: asexual spore production
Conidiophore: asexual reproductive structure
Cleistothecia: closed sexual fruiting bodies
Heterokaryon: hyphae carrying two different types of nuclei
Homokaryon: hyphae carrying nuclei of the one type
Homothallic: capable of sexual reproduction without a mating partner
Hyphae: filamentous vegetative growth tubes
Metulae: primary sterigmata, which bud from the conidiophore vesicle
Mycelia: vegetative hyphae
Phialides: secondary sterigmata, which bud from the metulae
Sterigmata: collective term for metulae and phialides

- *A. nidulans* is haploid, allowing easy selection of mutants and Mendelian analysis in a single generation. Meiotic genetics permits mapping of mutations and generation of progeny carrying multiple mutations, as detailed in the protocol below. Diploids can be formed and heterokaryons generated for complementation analysis and dominance tests, and mitotic genetics allows rapid mapping of newly identified genes to linkage groups, detection of translocations and mapping of centromeres using mitotic recombination[28].

Using *A. nidulans* for molecular genetic analyses

In addition to classical genetics, *A. nidulans* molecular genetics is well developed, and the combination is extremely powerful. DNA-mediated transformation is routine in *A. nidulans* and usually occurs via nonhomologous integration or by homologous integration[29–32]. Many selectable markers, including dominant selectable markers, are available, for example, *amdS*[29], *pyrG*[33], *riboB*[34], *pyroA*[35], *prn*[36], *argB*[37], *trpC*[38]. Gene targeting is possible using several selection systems, and mutant strains have recently been developed to achieve high levels of homologous integration[31,39–41]. Tightly regulated inducible promoters are available for overexpression or conditional expression[42,43], and RNA-mediated gene silencing can be used to downregulate gene expression[44]. Genes may be readily cloned by complementation using autonomously replicating AMA1-containing vectors[45,46].

Analysis of meiotic progeny

The segregation of mutant phenotypes in meiotic crosses allows one to determine whether a phenotype is due to a mutation in a single gene. Crosses involving multiple mutations can be used to map the mutations with respect to each other and also to construct strains with different combinations of mutations to examine the functional relationships between genes. *A. nidulans* provides an excellent system for genetic analysis and has the considerable advantage over diploid genetic systems of allowing genetic analysis and construction of multiply mutant strains in a single generation.

We describe a protocol for crossing *A. nidulans* strains by establishing a heterokaryon between two parent strains and incubating under conditions that allow initiation of the sexual (meiotic) cycle. We describe the isolation and cleaning of individual fruiting bodies (cleistothecia), which contain the meiotic progeny (ascospores), the generation of ascospore suspensions and testing for hybrid cleistothecia. We provide an example of analysis of meiotic progeny by growth testing.

MATERIALS

REAGENTS

- Strains: *A. nidulans* strains are available from the Fungal Genetics Stock Center (http://www.fgsc.net/). Storage of strains is described in **Box 3**
  ▲ CRITICAL Chemicals may be purchased from standard chemical supply companies.
- 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

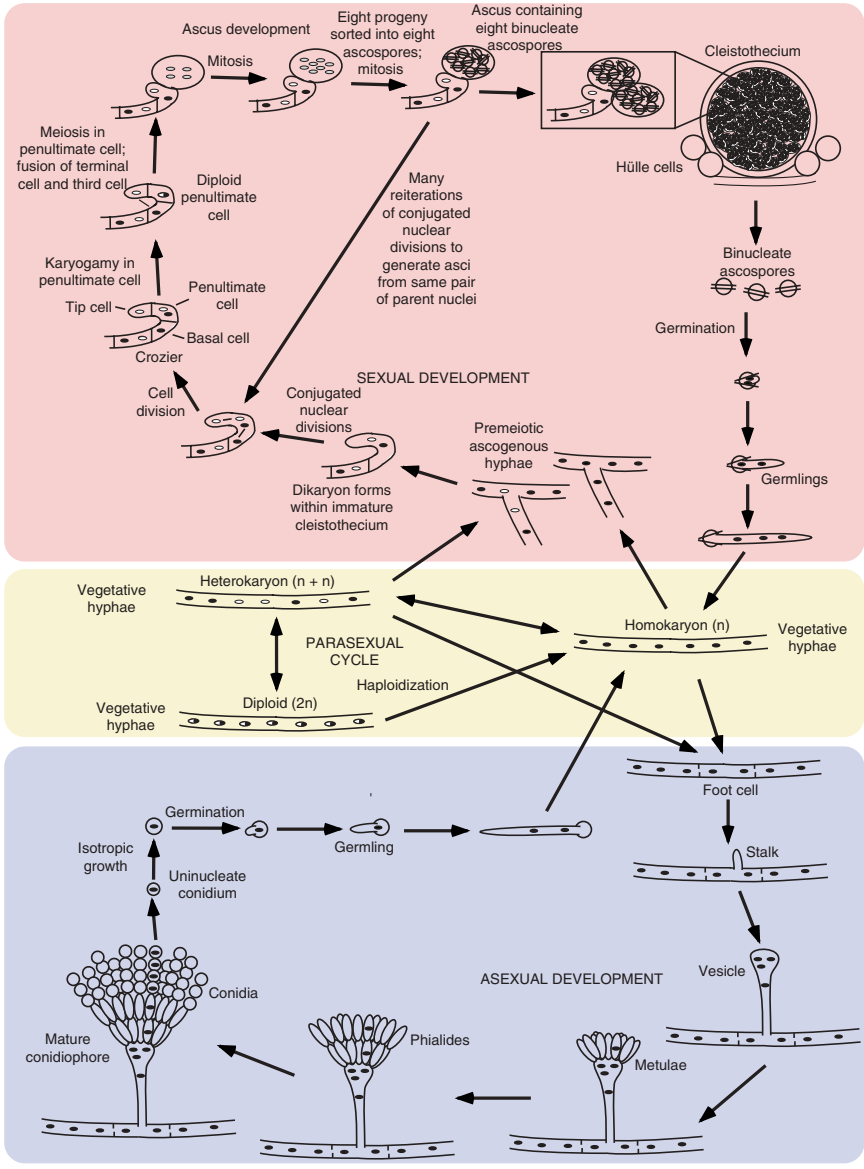

Figure 1 | Life cycle of *A. nidulans*. *A. nidulans* grows as haploid vegetative filamentous hyphae following germination of uninucleate conidia or binucleate ascospores. Vegetative hyphae from two individuals may fuse to form a heterokaryon and nuclei in a heterokaryon or a homokaryon may fuse to form a diploid (parasexual cycle, highlighted in yellow). Vegetative hyphae differentiate by asexual development (highlighted in purple) 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 more than 10,000 ascospores that are the meiotic progeny of a single ascogenous hypha[1]. Asci arise from croziers, which are formed by simultaneous division of the two nuclei of the terminal cell of an ascogenous hypha[48]. 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. Usually, 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[1]. The genetic crosses in this protocol use the sexual cycle of *A. nidulans*, which is outlined in the upper part of the figure (pink). Clonal propagation for growth testing and replica plating relies on the production of asexual conidia, shown in the lower part of the figure (purple).

- Riboflavin
- Casein hydrolysate
- D-Glucose
- Ammonium tartrate
- Peptone
- Yeast extract
- Agar
- Sodium nitrate
- Sodium hydroxide
- Skim milk powder
- Silica gel
- 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 and pyridoxine (see REAGENT SETUP)
- 4.0% water agar plates (see REAGENT SETUP)
- Sterile deionized water

EQUIPMENT

- Growth chambers (incubators) at 37 °C
- Double-wire twist replicator (**Fig. 2**)
- 26-prong double-wire twist replicator (**Fig. 2**)
- Plastic Petri plates (90 and 55 mm)
- Bunsen burner
- Microfuge tubes
- Dissecting microscope
- Masking tape

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.

BOX 2 | THE USE OF AUXOTROPHIC AND COLOR MARKERS IN *A. nidulans* MEIOTIC CROSSES

A. nidulans meiotic crosses

Crosses between two *A. nidulans* strains occur in a heterokaryon formed between the two parents. A heterokaryon may form cleistothecia containing progeny arising from selfing of either parental type or by formation of a hybrid cleistothecium. To maximize hybrid cleistothecium formation, a balanced heterokaryon carrying equal proportions of the parental nuclei is desirable. Balanced heterokaryons may be formed using parent strains that have complementary auxotrophic markers.

Auxotrophic markers

Prototrophs are strains that can grow on unsupplemented minimal medium. Auxotrophs are unable to grow on minimal medium without provision in the media of a particular nutrient they require owing to a mutation leading to inability to synthesize that nutrient. The inability of auxotrophs to grow on unsupplemented minimal medium can be used to force heterokaryons between two parents that have auxotrophic mutations affecting different genes. Heterokaryon formation may occur following transfer of mixed hyphae from the two parents to selective media lacking the particular nutrients required for each parent to grow. Growth of each auxotrophic parent is selected against. However, a balanced heterokaryon carrying nuclei from both parents is able to grow because the nucleus from each parent carries a wild-type copy of the gene that is defective in the nucleus from the other parent. Choosing strains carrying different auxotrophic markers as parents for crossing allows formation of a balanced heterokaryon carrying similar proportions of both parental nuclei and therefore increases successful formation of hybrid cleistothecia.

Distinguishing hybrid cleistothecia from cleistothecia derived from selfing

Auxotrophic markers are useful in identifying hybrid cleistothecia. Auxotrophic markers segregate in the progeny of a hybrid cleistothecium but not in the progeny from a cleistothecium arising from selfing and therefore can be used to distinguish hybrid cleistothecia on the basis of whether different combinations of the auxotrophic markers are present in the progeny. The presence of wild-type recombinant progeny within a cleistothecium is readily tested by plating a sample of the ascospore suspension on media selecting against both parental types. Ascospore suspensions from cleistothecia derived by selfing of either parent give progeny only of a parental type, which are unable to grow on selective media. Ascospore suspensions from hybrid cleistothecia yield wild-type recombinant progeny that grow on selective media.

Conidial color markers

The conidial color mutations of *A. nidulans* provide a very convenient way to assess by visual inspection on nonselective medium whether a cleistothecium is hybrid or arose from the selfing of one or other parent.

In a cross between a wild-type (green) conidial color strain and a yellow (*yA1*) mutant (as in the example in **Fig. 3**), cleistothecia arising from selfing of the wild-type parent only give rise to green progeny and cleistothecia arising from selfing of the *yA1* parent only give rise to yellow progeny. Hybrid cleistothecia contain progeny that form yellow conidia (because they inherited the *yA1* allele from the yellow parent) and progeny that produce green conidia (as they inherited the wild-type allele from the green parent).

In a cross between a yellow (*yA1*) mutant and white (*wA3*) mutant, hybrid cleistothecia contain green progeny, yellow progeny and white progeny. The *yA1* and *wA3* mutations are in different genes, which are unlinked. Therefore, the possible classes of progeny are *yA1 wA*$^+$ (yellow), *yA1 wA3* (white), *yA*$^+$ *wA3* (white) and *yA*$^+$ *wA*$^+$ (green). The *wA* mutation masks the effect of the *yA* mutation.

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 Ca salt), 50 mg pyridoxine·HCl, 100 mg riboflavin. Store wrapped in a 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 pH to 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 STEP 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 pH to 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. For selective media plates for crosses (Step 3), pour into thick small (55 mm) plates to minimize the oxygen present following sealing in Step 5. ! 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.

4.0% water agar plates (per liter) Add 4.0% (w/v) agar to deionized water. Autoclave. Allow to cool. Pour 20–25 ml media per 90 mm plastic Petri dish. Allow the media to set. Plates may be prepared weeks before use and stored at 4 °C.

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 nitrogen-free medium. Ammonium tartrate ($(NH_4)_2T$) and sodium nitrate (Na_2NO_3) are nitrogen sources that support strong growth.

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).

PROCEDURE

Establish a heterokaryon and allow crossing to occur

● TIMING **9–14 days**

1| Using a sterile wire twist, stab-inoculate the two parent strains 5 mm apart on a solid 1.0% complete medium plate (see REAGENT SETUP) (**Fig. 3**).

▲ CRITICAL STEP The parent strains must be inoculated close together to allow anastomoses (hyphal fusions) to occur. Colonies that are further apart than 5 mm may exhibit contact inhibition and the parents may not meet.

▲ CRITICAL STEP It is valuable to use parents carrying different conidial color markers—this allows hybrid cleistothecia to be easily distinguished from cleistothecia arising from selfing of either parent at Step 12 (see **Box 2**). Crosses between strains carrying auxotrophic markers but with the same conidial color may be undertaken and hybrid cleistothecia identified by the presence of wild-type recombinant progeny on selective media at Step 12 (see **Box 2**).

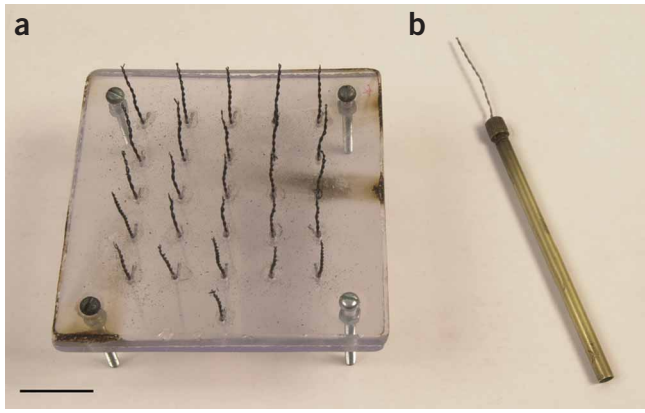

Figure 2 | Tools for physical transfer of *A. nidulans* conidia. (**a**) 26-prong double-wire replicator, used for replica plating conidia from a master plate of 26 colonies to various test media. (**b**) Double-wire replicator, used for transfer of conidia from a single colony to growth media. Scale bar, 20 mm.

■ PAUSE POINT *A. nidulans* is a very convenient research organism as it 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. *A. nidulans* will resume growth upon return to 37 °C. Ascospores may be stored for several years at room temperature; however, differing viability may be observed for some markers. Further storage instructions are provided in **Box 3**.

2| Incubate for 2 days at 37 °C (**Fig. 3a**).

? TROUBLESHOOTING

3| Using a sterile wire twist, cut out the freshly growing non-conidiating edge of the colony where the two parents meet (**Fig. 3b**) and transfer to a small plate containing solid selective medium that lacks nutrients required for each of the parental strains, allowing selection against growth by the parents but allowing growth of a heterokaryon (**Fig. 3c**).

4| Incubate for 2 days at 37 °C.

5| Seal the plate with masking tape to exclude oxygen.

▲ CRITICAL STEP Allow growth for 2 days to establish heterokaryotic growth before sealing the plate. *A. nidulans* is an obligate aerobe and will not grow if oxygen is excluded. Exclusion of oxygen promotes cleistothecia formation.

6| Incubate for 5–10 days until cleistothecia are observed (**Fig. 3d,e**).

At this stage, cleistothecia usually contain sufficient numbers of progeny for most purposes and one may proceed directly to Step 7. However, should large numbers of progeny be required, one may wait for the cleistothecia to fully ripen for an additional 2 weeks, giving more than 100,000 viable ascospores per cleistothecium.

? TROUBLESHOOTING

Preparation of ascospores from cleistothecia ● TIMING 5–10 min per cleistothecium

7| Using a sterile single wire, pick mature cleistothecia and transfer to a 4% water agar plate (**Fig. 3f**). Note that when crossing strains of different conidial color, hybrid cleistothecia are more likely to occur in heterokaryotic regions of the colony containing conidia of both colors than in unbalanced heterokaryotic regions containing only one conidial color.

▲ CRITICAL STEP Some cleistothecia will be the products of selfing of one or other parent and some cleistothecia will be hybrids. It is often necessary to test several cleistothecia to find a hybrid cleistothecium. We usually clean and test four cleistothecia per cross in the first instance—if no hybrid cleistothecia are among the first four cleistothecia tested, additional cleistothecia need to be cleaned and examined.

▲ CRITICAL STEP Do not discard the crossing plate containing cleistothecia (**Fig. 3d**)—these may be required if the chosen cleistothecia are not hybrids.

8| View the cleistothecium using a dissecting microscope. Clean the cleistothecium from hyphae, Hülle cells and conidiophores by rolling the cleistothecium along the surface of the agar plate using a sterile wire twist (**Fig. 3g,h**). Sterilize the wire twist periodically during cleaning.

? TROUBLESHOOTING

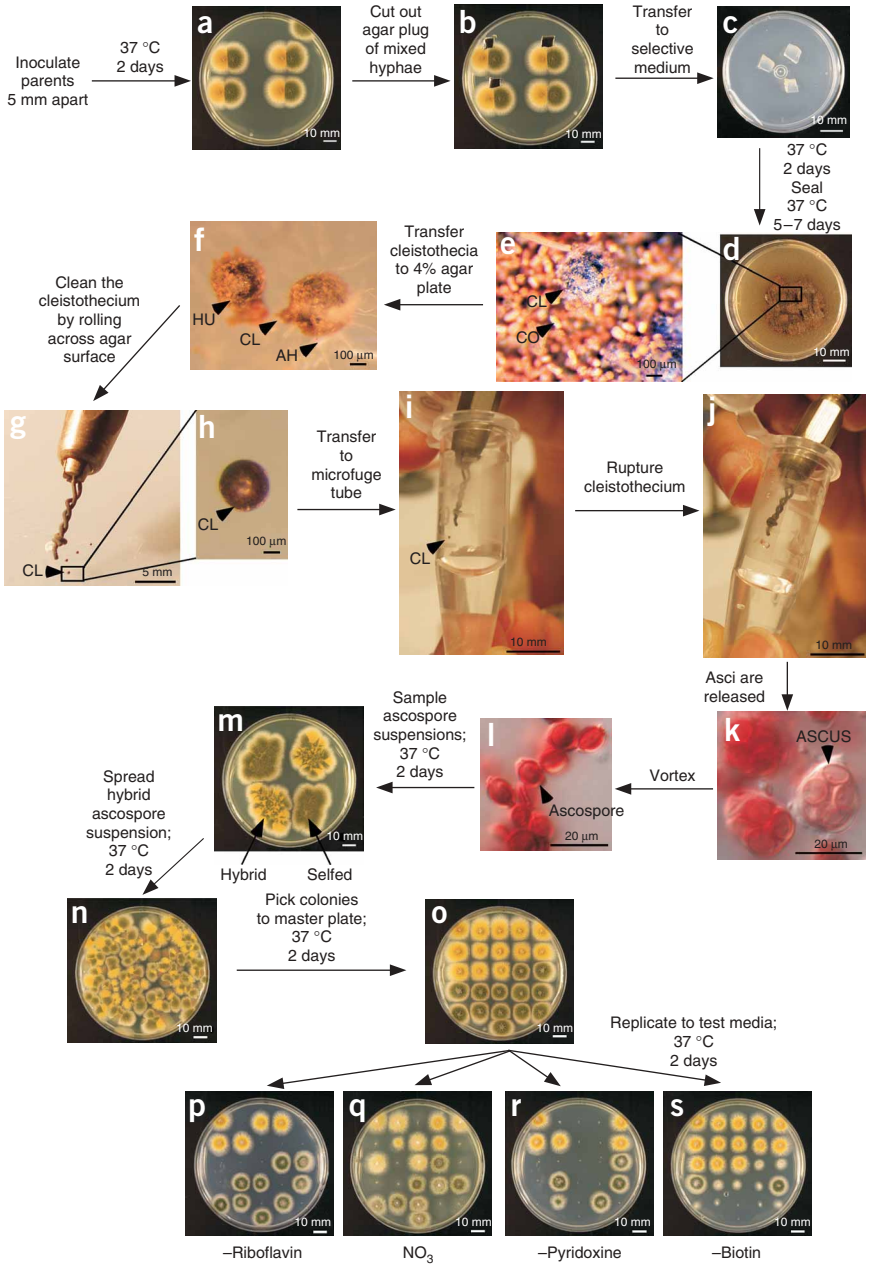

Figure 3 | Sexual crossing of *A. nidulans*. (**a**) The two parent strains are inoculated 5 mm apart on a 1.0% complete medium plate and incubated for 2 days at 37 °C. (**b**) An agar plug of mixed hyphae is cut out and transferred to selective medium (**c**), incubated at 37 °C for 2 days, sealed with masking tape and incubated for a further 5–7 days (**d**). The *A. nidulans* fruiting bodies (cleistothecia (CL)) appear on a bed of conidiophores (CO) (**e**) and are transferred with a sterile wire to a 4% agar plate. The cleistothecia are enveloped with Hülle cells (HU) and aerial hyphae (AH) (**f**). Hülle cells, aerial hyphae and any conidiophores are removed by rolling the cleistothecium across the agar plate with a sterile wire (**g**) until the cleistothecium is clean (**h**). The cleistothecium is transferred to a sterile microfuge tube containing 500 µl sterile water (**i**) and ruptured against the wall of the tube with the wire (**j**) to release the asci (**k**). The asci are disrupted by vortexing to generate an ascospore suspension (**l**). Ascospore suspensions are sampled by plating to complete medium to determine whether they arose from a hybrid cleistothecium or from selfing of either parent (**m**). An ascospore suspension arising from a hybrid cleistothecium is then spread plated (**n**) and colonies are transferred to a master plate (**o**). The master plate may then be replicated to various test media. Twenty-six progeny are shown from the cross MH54 (*biA1 niiA4*) × MH50 (*yA1 pyroA4 riboB2*). The markers are as follows: *biA1,* biotin auxotrophy; *niiA4,* nitrite utilization; *yA1,* yellow conidia; *pyroA4,* pyridoxine auxotrophy; *riboB2,* riboflavin auxotrophy. Conidial color may be scored by visual inspection. The test media shown are as follows: (**p**) ANM + $(NH_4)_2T$ + pyridoxine + biotin (i.e., lacking riboflavin; for scoring *riboB2*), (**q**) ANM + NO_3 + pyridoxine + biotin + riboflavin (for scoring *niiA4*), (**r**) ANM + $(NH_4)_2T$ + biotin + riboflavin (i.e., lacking pyridoxine; for scoring *pyroA4*) and (**s**) ANM + $(NH_4)_2T$ + pyridoxine + riboflavin (i.e., lacking biotin; for scoring *biA1*). The biotin-requiring *biA1* mutant progeny grow with a range of phenotypes from no growth to poor growth depending on their proximity to wild-type biotin-producing colonies, which secrete biotin into the medium and allow cross-feeding. All the *biA1* progeny in the sample shown exhibit wild-type conidial color because *biA1* is tightly linked to the *yA* locus.

9| Transfer the cleaned cleistothecium using a freshly sterilized wire twist to 500 µl sterile water in a microfuge tube (**Fig. 3i**). Rupture the cleistothecium by squashing against the side of the tube (**Fig. 3j**). Red ascospores in asci will be released (**Fig. 3k**). Vortex the ascospore suspension vigorously (**Fig. 3l**).
▲ CRITICAL STEP It is easiest to rupture the cleistothecium against the side of the tube in a small droplet of water and then mix the ascospores into a larger volume.

Identification of hybrid cleistothecia ● TIMING 2–3 days

10| Test whether the cleistothecium was formed by selfing of one or other parent or whether it is a hybrid by plating a loop-full (or 5 µl) of the ascospore suspension on solid complete medium (if color markers are segregating in the cross) or on solid selective medium to determine whether recombinant progeny are present. Note that variation in the frequency of hybrid cleistothecia occurs depending on the genotypes of the parents.

11| Incubate for 2–3 days at 37 °C.

BOX 3 | STORAGE OF *A. nidulans*

Short-term storage: *A. nidulans* strains may be stored for several months on solid media slopes in small screw-cap vials at 4 °C
▲ CRITICAL Aerobic growth in vials requires caps to be loosened during growth (usually 2 days at 37 °C) and then tightened for storage.

Long-term storage: *A. nidulans* strains may be stored for many years on silica gel.
1. Resuspend conidia from freshly grown slopes in a sterile 5% aqueous solution of skim milk. Alternatively, conidial suspensions may be made in sterile water.
2. Pipette the conidial suspension into a microfuge tube containing dry sterile silica gel.
3. Allow the tube to dry completely at room temperature. Vortex vigorously before storage at 4 °C.
▲ CRITICAL Store silica gel stocks in an air-tight container containing loose silica to prevent rehydration.

12| Inspect the plates to determine whether the progeny were derived from a hybrid or selfed cleistothecium. Hybrid cleistothecia contain progeny segregating for different markers whereas cleistothecia arising from selfing of one or other parent only contain progeny of the same genotype as that parent. In crosses in which conidial color markers are segregating (see **Box 2**), a hybrid cleistothecium contains progeny of different conidial colors, whereas a cleistothecium arising from selfing of one or other parent contains progeny of a single conidial color. In crosses between strains with auxotrophic markers of the same conidial color, hybrid cleistothecia are distinguished from those arising from selfing of one or other parent by plating on media selecting against both parental types. Progeny arising from selfings of either parent will not grow on selective media, whereas the wild-type recombinant progeny from hybrid cleistothecia will grow on selective medium.

Isolation of progeny for analysis ● TIMING 2–3 days

13| Choose an ascospore suspension (from Step 9) from a hybrid cleistothecium (**Fig. 3m**), vortex and spread at low colony density. Dilute 2–3 µl in 100 µl sterile H_2O and spread on a complete media plate.
▲ CRITICAL STEP The ascospore concentration may vary. It may be necessary to plate a greater or lesser volume. Normally, a plating density giving well-separated colonies (less than approximately 50 per plate) ensures that picking colonies for genotyping is not biased by different rates of colony growth.
? TROUBLESHOOTING

14| Incubate for 2–3 days at 37 °C (**Fig. 3n**).

Analysis of progeny by growth tests ● TIMING 4–5 days

15| Transfer progeny to a master plate (complete medium) for analysis (see **Box 4B**).

BOX 4 | GROWTH TESTS

The conidia of *A. nidulans* can be readily transferred on wire twists (**Fig. 2**) to solid media. The relative growth of the discrete compact colonies can then be assessed.

(A) For growth tests of a small number of strains, inoculate directly to each test medium
1. Make a single stab inoculum of each strain on the test media using a sterilized double-stranded twist of nichrome wire.
▲ CRITICAL STEP It can be extremely useful to include controls of known genotype. This checks that the medium is correct and provides a known comparitor for scoring of mutant phenotypes.
2. Incubate at 37 °C for 2–3 days or longer depending on the mutant phenotype.

(B) For growth tests of a large number of strains, replicate colonies to appropriate test media from a master plate
1. Set up a master plate containing 26 colonies arranged in a grid (1.25 cm apart) on complete media.
▲ 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. This checks that the medium is correct and provides a known comparator for scoring of mutant phenotypes.
2. Incubate for 2–3 days at 37 °C (**Fig. 3o**).
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 (**Fig. 2**). The wire twists are arranged in the pattern of the master plate grid.
Incubate the replica plates. Growth tests are usually (but not always, e.g., temperature-sensitive mutants) carried out at 37 °C. The plates are usually scored after 2 or 3 days incubation (see ANTICIPATED RESULTS). Colony growth may be photographed (**Fig. 3p–s**) or scored using symbols (**Table 2**). Where cross-feeding of nutrients from neighboring colonies occurs (e.g., for biotin-requiring progeny located near biotin prototrophs (**Fig. 3s**)), growth may be more accurately scored after 1 day's growth.

Although generally unnecessary, progeny may be picked with the aid of a dissecting microscope, allowing the use of a small inoculum and reducing the chances of colony cross-contamination. Use of a dissecting microscope allows slow-growing segregants to be more readily visualized.

16| Incubate for 2 days at 37 °C (**Fig. 3o**).

17| Replicate to selective media for phenotypic analysis and score according to the markers segregating in the cross (**Fig. 3p–s**) (see **Box 4B**).

● TIMING

The whole procedure can be completed in 2.5–3 weeks, including analysis of progeny.

? TROUBLESHOOTING

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

TABLE 1 | Troubleshooting table.

Step	Problem	Solution
1–17	Contamination	Ensure medium is sterile; work using Bunsen burner
2	Strains have not grown	(a) Check medium 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
6	Cleistothecia have not formed	Check genotypes of parent strains. Some mutants are self-sterile and are unable to form cleistothecia. Some of these mutants fail to form cleistothecia in homozygous crosses (e.g., some sexual development mutants, some auxotrophs such as the *riboB2* mutant) and therefore two strains containing these mutations cannot be crossed. Crosses between two self-sterile parents with different sterility mutations may generate hybrid cleistothecia but not selfed cleistothecia
8	Cleistothecia burst during cleaning	The cleistothecia may not be mature—incubate further to allow the cleistothecial wall to harden Mature cleistothecia are easier to roll clean than immature cleistothecia. Mature cleistothecia are those with a hard deep red (almost black) wall. Immature cleistothecia are pale red-pigmented and are easily broken
12	No progeny grew on the test plate	(a) The cleistothecium may not have been ruptured sufficiently in Step 9. Ensure the cleistothecium is ruptured, vortex the ascospore suspension vigorously (b) The cleistothecium may be barren—choose another cleistothecium (c) Check that the test media contained all supplements required for growth and a nitrogen source
12	No hybrid cleistothecia were formed	Go back to Step 7 and clean additional cleistothecia
12	No hybrid cleistothecia were formed; all cleistothecia were derived from one parent	By chance the heterokaryon formed was not well balanced and predominately contains nuclei from one parent. Consequently, hybrid cleistothecia are rare. This is often observed when the parent strain(s) only contain leaky markers (e.g., *biA1*), which do not provide strong selection against the parent. Go back to Step 7 and clean additional cleistothecia or go back to Step 1 and set up a new heterokaryon
17; **Box 4B**, Step 3	All progeny are parental types (no recombinant progeny) even though the cleistothecium appeared hybrid on the basis of progeny of both conidial colors	The cleistothecium chosen was a twin cleistothecium containing progeny arising from fusion of selfed cleistothecia from each parent. Twin cleistothecia are obvious by the complete absence of recombinant progeny. Choose another cleistothecium

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Solution
17; **Box 4B**, Step 3	Aberrant segregation ratios among progeny	Aberrant ratios may arise if a particular genotype confers reduced viability or if the medium used for plating was deficient for a growth requirement. Check that the medium contains supplements required for growth of all progeny
17; **Box 4B**, Step 3	Aberrant segregation ratios among progeny—excess of one parental type	(a) The cleistothecium may not have been sufficiently cleaned. (b) The cleistothecium chosen was a twin cleistothecium containing progeny arising from fusion of a selfed and a hybrid cleistothecium Choose another cleistothecium
17; **Box 4B**, Step 3	Aberrant segregation ratios among progeny—apparent excessive wild-type alleles segregating	(a) This may arise from an excess of mixed colonies transferred to the master plate due to the plating density being too high Plate at lower colony density (Step 13) (b) Mixed colonies may arise due to ascospores sticking together Vortex the ascospore suspension thoroughly before plating (Step 13)

ANTICIPATED RESULTS

Haploid Mendelian recombination of genes or linkage analysis in one generation is expected.

An example of ANTICIPATED RESULTS is shown in **Figure 3** for the cross between MH54 (*biA1 niiA4*) and MH50 (*yA1 pyroA4 riboB2*). The phenotypes are scored as follows: growth in the absence of riboflavin for *riboB*$^+$ versus no growth for *riboB2* (**Fig. 3p**), growth utilizing nitrate as the sole nitrogen source for *niiA*$^+$ versus no growth for *niiA4* (**Fig. 3q**), growth in the

TABLE 2 | Inferred genotypes of *A. nidulans* progeny from growth tests.

Test media	Complete[a]	NO_3[b]	−Pyridoxine[c]	−Riboflavin[d]	−Biotin[e]	
Marker	***yA1***	***niiA4***	***pyroA4***	***riboB2***	***biA1***	**Inferred genotype**
Colony number[f]						
1	Y	+	+	+	+	*yA1*
2	Y	+	-	-	+	*yA1 pyroA4 riboB2*
3	Y	-	-	+	+	*yA1 pyroA4 niiA4*
4	Y	+	-	+	+	*yA1 pyroA4*
5	Y	+	+	-	+	*yA1 riboB2*
6	Y	-	+	+	+	*yA1 niiA4*
7	Y	+	+	+	+	*yA1*
8	Y	+	-	-	+	*yA1 pyroA4 riboB2*
9	Y	+	-	-	+	*yA1 pyroA4 riboB2*
10	Y	-	+	-	+	*yA1 niiA4 riboB2*
11	Y	+	-	-	+	*yA1 pyroA4 riboB2*
12	Y	-	-	-	+	*yA1 pyroA4 niiA4 riboB2*
13	Y	+	-	-	+	*yA1 pyroA4 riboB2*
14	G	+	-	+	-	*biA1 pyroA4*
15	G	-	+	+	-	*biA1 niiA4*
16	G	-	-	-	+	*pyroA4 niiA4 riboB2*
17	G	-	+	+	-	*biA1 niiA4*
18	G	+	-	+	-	*biA1 pyroA4*
19	G	+	-	-	-	*biA1 pyroA4 riboB2*
20	G	+	+	+	+	WT
21	G	+	-	+	-	*biA1 pyroA4*
22	G	+	+	+	-	*biA1*
23	G	+	-	-	-	*biA1 pyroA4 riboB2*
24	G	-	+	+	-	*biA1 niiA4*
25	G	-	-	-	-	*biA1 pyroA4 niiA4 riboB2*
26	G	+	-	+	-	*biA1 pyroA4*

[a] Complete medium (**Fig. 3o**) allows all progeny to grow and the conidial color to be scored as green (G) (*yA*$^+$) or yellow (Y) (*yA1*). [b] ANM plus NO_3, pyridoxine, riboflavin and biotin medium allows growth (*niiA*$^+$) versus no growth (*niiA4*) utilizing nitrate as the sole nitrogen source to be scored (**Fig. 3q**). [c] −Pyridoxine (ANM plus NH_4, riboflavin and biotin) medium allows growth (*pyroA*$^+$) versus no growth (*pyroA4*) to be scored (**Fig. 3r**). [d] −Riboflavin (ANM plus NH_4, pyridoxine and biotin) medium allows growth (*riboB*$^+$) versus no growth (*riboB2*) to be scored (**Fig. 3p**). [e] −Biotin (ANM plus NH_4, pyridoxine and riboflavin) medium allows growth (*biA*$^+$) versus no growth (*biA1*) to be scored (**Fig. 3s**). [f] The colony number corresponds to the colonies on the master plate and replica plates in **Figure 3p–s** from left to right as follows: row 1 = colony numbers 1–5; row 2 = colony numbers 6–10; row 3 = colony numbers 11–15; row 4 = colony numbers 16–20; row 5 = colony numbers 21–25; row 6 = colony number 26.

absence of pyridoxine for *pyroA*$^+$ versus no growth for *pyroA4* (**Fig. 3r**) or strong growth in the absence of biotin for *biA*$^+$ versus no growth or poor growth (owing to cross-feeding) for *biA1* (**Fig. 3s**). The mutant phenotypes may be recorded using photographs (as in **Fig. 3**) or tabulated using symbols to score growth, and the genotypes inferred (**Table 2**).

To examine for segregation of alleles, the number of each phenotypic class is counted. For example,

13 *yA*$^+$ (green): 13 *yA1* (yellow) (**Fig. 3o**)
14 *riboB*$^+$: 12 *riboB2* (**Fig. 3p**)
15 *biA*$^+$: 11 *biA1* (**Fig. 3s**)

Therefore, each of these markers segregates in the expected 1:1 ratio for allelic genes. Departures from 1:1 segregation may occur in the case of poor viability of one class of progeny or non-Mendelian inheritance. Standard χ^2 analysis can be used to test statistical significance[47].

To examine for independent assortment of genes, the number of each phenotypic class is examined. For example, there is approximately 1:1 segregation of *riboB2* and *riboB*$^+$ among the yellow progeny and approximately 1:1 segregation of *riboB2* and *riboB*$^+$ among the green progeny (**Fig. 3p**). Therefore, *yA* and *riboB* show independent assortment. In contrast, all the yellow progeny are *biA*$^+$ and 11/13 of the green progeny are *biA1*. This indicates linkage between the *biA* and *yA* loci. Standard χ^2 analysis can be used to test statistical significance[47].

For three-point mapping crosses, the relative order and map distances of the three genes may be determined[1].

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