How to sample natural populations.

Background

Strains from nature have been widely used in experimental and evolutionary studies (reviewed by Perkins and Turner 1988, Turner *et al*. 2001), most recently for creating phylogenetic trees which provide information on speciation (Dettman *et al.* 2003). Natural populations have usually been sampled by obtaining conidia from visible colonies on burned vegetation, mostly in tropical or subtropical regions. Soil samples have been another source, yielding mainly ascospore-derived homothallic and pseudohomothallic isolates. Most recently, trees killed by forest-fires have provided a source in temperate climates (Jacobson *et al.* 2004). Wild populations have been the only source of Spore killer strains (Turner 2001).

Procedure for visible vegetative colonies

Systematic sampling of wild populations began in 1968, using methods described by Perkins *et al.* (1976). The procedures have remained largely unchanged. Small, autoclaveable 'glassine' envelopes are used for collecting. (The needle-sterilizing envelopes originally specified are no longer available. 45 × 74 mm envelopes from philatelic supply sources are now used (e.g., http://www.brooklyngallery.com). A strip of chromatography paper or filter paper (~5 × 45 mm) is placed in each envelope, together with a wood toothpick. (The toothpick may be found unnecessary.) These are autoclaved, dried, and enclosed as bundles of ten in larger polyethylene bags or envelopes (~10 × 15 cm). Supplies for a day's collecting are easily carried in a pocket.

In the field, samples of conidia from individual Neurospora colonies are placed on the paper strips, either by touching directly or by using the toothpick for transfer. Dry conidia are best for retaining viability. Each small collecting envelope is closed and labeled with details of locality, substrate, date, etc. If desired, 20 or more sealed collection envelopes can be taped to a paper sheet, enclosed in a first-class letter envelope, and airmailed to the home laboratory. For bringing stocks into the United States, an import permit can be obtained from the U.S. Department of Agriculture, Animal and Plant Health Inspection Service (http://www.aphis.usda.gov/ppq/permits/).

Upon receipt in the laboratory, shipments are placed at –20ºC for at least 24 hours before subculture. Mite eggs and adults are killed at this temperature, but Neurospora conidia are not. A sterile transfer needle is used to remove conidia or a small section is cut from the filter paper strip with sterile scissors and transferred to slants of minimal medium plus chloramphenicol (200 μg/ml, autoclavable) to inhibit bacteria. Conidia from each transferred culture are streaked on water-agar or sorbose minimal medium and one colony of single-conidial origin is used to establish a stock culture which is assigned an accession number and used for further tests to establish mating type, species, etc. as described in *How to determine the species of a wild-collected isolate*. The collecting envelope can be resealed and stored indefinitely at –20ºC.

Collecting strategy. Material is collected by preference from small discrete colonies growing on recently burned vegetation, thus minimizing the obtaining of clonal derivatives of identical genotype. Seven to ten colonies should be sampled at each collection site to assure viability and to obtain both mating types.. Larger samples may be desired to reveal genetic polymorphisms or the presence of more than one species. Neurospora is also found conidiating under the bark of fire-damaged trees following forest fires in western North America, in cavities where heat of the fire has resulted in separation of the bark from inner woody layers (Jacobson *et al.* 2004).

Procedure for soil samples

The following procedure (adapted from Glass *et al.* 1990) depends on germinating dormant ascospores that are present in the soil. Activated ascospores of heterothallic and pseudohomothallic species grow up and produce conspicuous orange conidia. Mycelia from ascospores of homothallic species do not conidiate but they do produce perithecia. Ascospores from these are a ready source of pure homothallic cultures. Heterothallic species will not produce perithecia on the plate unless ascospores of opposite mating type were also present in the same soil.sample.

Samples of about 5 or 10 g are taken from the upper 1 or 2 cm. of soil and put into small plastic ('Ziploc') bags, which are marked and sealed en masse into a larger bag. The bags may be stored at 4ºC until they are sampled for isolation of fungi. (Freezing is likely to kill ascospores and should be avoided.) All soils brought into the United States must enter under a permit issued by the Department of Agriculture, and must be handled with preapproved containment procedures.

Media for germination and sporulation are prepared by autoclaving the following together to make 1 liter: D-xylose, 10 g; agar, 10 g; KNO_3, 1 g; KH_2PO_4, 875 mg; K_2HPO_4; 160 mg; $MgSO_4$.7 H2O, 500 mg; NaCl, 100 mg; $CaCl_2$, 100 mg; biotin, 5µg; and trace elements at the concentrations used for minimal medium N or for synthetic cross medium. Chloramphenicol (300 mg) and 2-furyl alcohol (1 ml of 10% aqueous solution) are added when the medium has cooled to 60ºC. Growth of bacteria but not of Neurospora is inhibited by the low pH and the antibiotic. Ascospores are activated by the furyl alcohol and the high temperature. Unlike furfural, the 2-furyl alcohol is not toxic at the concentration used. Xylose gives better germination than sucrose.

25 ml of the medium at 60ºC is added to each soil sample (typically about 2 g) in a 50-ml screwcap tube. Tubes are shaken vigorously a few times and held at 60ºC for 20 min. They are then shaken to resuspend the soil. The contents are poured into petri dishes and incubated at room temperature.

Within 2 or 3 days, vegetative colonies of Neurospora and other fungi will become visible. If desired, vegetative colonies.can be sampled by making transfers from the isolation plates to tubes of chloramphenicol-supplemented minimal medium N or synthetic cross medium. This should be done early, to avoid obtaining mixed cultures, especially of conidiating species. The tubed cultures are incubated to test for self-fertility, and are further purified and identified to species as described in *How to determine the species of a wild-collected isolate.*

Perithecia are visible on isolation plates within a week, and ascospores will have been ejected onto the lids after 2 or 3 weeks. Mature ascospores that were ejected onto the petri dish lid are collected in a few drops of sterile distilled water to which a drop of glycerol has been added. A drop of spore suspension is placed on a microscope slide, covered with a No. 1 coverglass, and examined at 500× to observe surface ornamentation and measure ascospore size. Neurospora ascospores are grooved, Gelasinospora spores are pitted, and those of Sordaria are smooth. The medium was designed to optimize germination of ascospores, optimize formation of fertile perithecia, minimize the canopy of vegetative growth of other fungi, inhibit the growth of bacteria, and kill mites, insects, and nematodes which might be present in the soil (Glass *et al.* 1990). Neurospora ascospores from each lid are isolated to small slants of minimal synthetic cross medium. Self-fertile cultures are examined to determine whether they are homothallic, with eight spored asci, or pseudohomothallic, with four-spored asci. Unfertilized f_1 cultures of heterthallic species do not produce perithecia. They are keyed out by crossing to species-tester strains as described in *How to determine species*.

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