Neurospora from Natural Populations: A Global Study

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The genus Neurospora includes five conidiating species: N. crassa, N. intermedia, N. sitophila, N. discreta (all heterothallic), and N. tetrasperma (pseudohomothallic). N. crassa is best known and has been used extensively for genetic, biochemical, and molecular studies (Davis, 2000). (The known homothallic species are all nonconidiating and are outside the scope of this study.) Systematic sampling of natural populations of the conidiating species was started in 1968. The program was described and early results were presented in Perkins et al. (1976). A comprehensive review (Perkins and Turner, 1988) presented the rationale, described the methods used for collecting and analyzing cultures, and enumerated the field-collected strains. It also summarized information on ecology, geographical distribution, species status, genetic variation, and polymorphism. Experimental laboratory projects that made use of wild-collected strains were described.

We now present a final report from this laboratory, giving a description of the complete collection, including geographical origins of strains of each species. In the Discussion, we survey advances since the 1988 review, summarizing new information regarding natural populations that has been obtained and describing new ways in which the field-collected strains have been used. Continued access to the collection will be provided by the Fungal Genetics Stock Center (FGSC, Department of Microbiology, University of Kansas Medical Center, Kansas City, KS), who have assumed responsibility for the stocks.

This long-term study has had two objectives: (1) to advance knowledge of the natural history, distribution, population structure, and systematics of Neurospora and (2) to provide genetic variants for laboratory investigations. Knowledge at the time of our last report (Perkins and Turner, 1988) is summarized in the following paragraphs.

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Individual Neurospora colonies found on recently burned vegetation are usually unique in genotype. Cultures obtained from undisturbed single colonies are usually monoclonal and unmixed. Mixed cultures of different mating types or different species can be purified by the subculture of single conidia. Thus, despite the potential for clonal propagation, these colonies provide effective population samples comparable to those collected for higher plants and animals, but with the advantage that the haploid, gametic generation is sampled.

The five conidiating Neurospora species cannot be distinguished from one another on the basis of vegetative morphology or color. Colonies and cultures are typically orange or orange-yellow. Mating-based criteria for the defining of species were established, and species-tester strains that were derived from authenticated type strains or were shown to be conspecific with them were developed. The species of a newly isolated haploid culture has been determined by it being crossed to a series of species-tester strains and the mating reaction being observed. Results with these testers were in reasonable accordance with those based on conventional criteria. Discovery of N. discreta brought to five the number of conidiating Neurospora species. Isolates from different geographic areas were assigned to the new species, N. discreta, spora species. Isolates from different geographic areas (spore morphology.) with the exception of matings that involve segments can be transferred from one species to another, nevertheless sufficiently fertile that genes and chromosome very rare in the laboratory. Interspecific crosses are nevertheless specific combinations, production of viable ascospores is found growing side by side in the field. For most interspecific combinations, production of viable ascospores is very rare in the laboratory. Interspecific crosses are nevertheless sufficiently fertile that genes and chromosome segments can be transferred from one species to another, with the exception of matings that involve N. discreta. Crosses between the closely related sibling species N. crassa and N. intermedia produce abundant ascospores, but these are mostly unpigmented and inviable.

Karyotypes, karyogamy, and meiotic chromosome behavior are similar for all the known Neurospora species, with seven chromosomes and a single terminal nucleolus organizer. (Karyotypes are also similar in several truly homothallic eight-spored lines that their discoverers designated different Neurospora species on the basis of ascospore morphology.)

Isozyme polymorphism in local populations of N. intermedia is comparable to that in outbreeding higher animals and plants. Restriction fragment length polymorphisms (RFLPs) are abundant in N. crassa, where they have been used for mapping cloned DNA segments. Genes governing vegetative (heterokaryon) incompatibility are highly polymorphic, as are recessive genes that adversely affect one or more stages of the sexual diplophase. Chromosomally located meiotic drive factors called Spore killer were discovered in populations of N. sitophila and N. intermedia. Mitochondrial genomes of strains from nature differ in both length mutations and nucleotide substitutions. Many isolates contain mitochondrial plasmids. A few strains undergo senescence following insertion of plasmid sequences into mitochondrial DNA.

**MATERIALS AND METHODS**

**Procedures for Processing Collected Cultures**

Culture media and standard procedures for handling Neurospora are given in Perkins (1959), Davis and de Serres (1970), and Davis (2000). Routine procedures for wild-collected cultures were as described by Perkins et al. (1976) and Perkins and Turner (1988), with some modifications given in the present paper. In brief, small envelopes, each containing a conidial sample that had been smeared on a sterile filter-paper strip, were placed at −20°C for at least 24 h to kill mites. Cultures were grown on minimal medium with chloramphenicol added to kill bacteria. Two successive transfers (using conidia from the top of the slant) were made to slants of minimal medium. Cultures were then preserved on anhydrous silica gel. Duplicate stocks were also kept on 10 × 75-mm slants at −20°C.

Collected samples are called "cultures" because some samples yield more than one strain. If cultures were purified or if additional results indicated that they were not mixed, they are usually referred to as "strains." We strongly advise that cultures be purified by conidial plating before they are used in laboratory investigations. Strains in the collection that have been derived from a potentially mixed culture by ascospore isolation or conidial plating are listed and identified as such in the FGSC web site. These strains have been given new identification numbers ("P" numbers) to distinguish them from the cultures from which they originated.

The successive transfers following isolation were adopted to eliminate fungal contaminants of other genera.
This procedure usually was effective, as judged by later sampling of the silica gels. Each transfer was examined for blooms of fast-growing fungi, such as M. oryzae, and for dots of slower-growing fungi. If fast-growing contaminants were detected, cultures were purified by the plating of very dilute suspensions of conidia on sorbose medium (Davis and de Serres, 1970; D. Davis, 2000). Slow-growing contaminants could usually be eliminated by use of additional transfers and close examination of the slants at 60× magnification. Slants of complete medium on 13 × 100-mm tubes were used for growth of the conidia to be placed on silica gel. These slants were also examined before and after processing, and the change in growth conditions sometimes revealed a previously undetected contaminant. Silica gels found to be made from contaminated cultures were discarded and replaced after the culture was purified, except that in several cases the contaminated Neurospora culture happened to be redundant for that species and region, so purification of the culture was put off until such time as additional cultures might be requested for that region. The silica gel was kept and marked “contaminant,” with a similar notation added to the record for that culture. In a small number of cases, a spore from an undetected contaminant of another genus could have survived undetected in a silica gel of our collection or even in the FGSC collection, which has passed through several further transfers.

A problem of much higher probability is the admixture of two Neurospora strains. Our initial processing was not designed to resolve cultures containing more than one Neurospora strain. On the contrary, we hoped to detect and save all of the Neurospora components in each culture. As explained in the section on determination of species, mixtures of various kinds were detected when cultures were crossed to the species-testers, but certain combinations would not have been detected, particularly if there were two strains with the same species and mating type.

At the time silica gel stocks were made, wild-collected cultures that were processed in this laboratory were given identification numbers preceded by the letter P. Most cultures with P numbers were collected by the second author, but some were collected by others and sent to us for processing and deposit. In some cases the collectors had already obtained conidial isolates from mixed cultures but had not ascertained their species. In the cases of contributed cultures derived from nonburned sources, particularly from soil samples, silica gels were not made nor P numbers assigned before the very heterogeneous original cultures were separated.

Thirty-six cultures among all those from burned vegetation were found to contain strains of more than one species. The original mixed cultures are all represented by silica gels and P numbers. Most were processed to obtain separate cultures of the components, which were then given their own P numbers. Among cultures that contained strains of both mating types of the same species, many came from sites where isolates comparable to both components were already available, but 56 cultures were separated into one strain of each mating type, and the separate strains were given P numbers. Also, about 20 f1 strains from these mixed-mating-type cultures were given P numbers during the early years of the project. Because N. tetrasperma is usually found as a culture containing both mating types, we usually did not separate the components, but for the few that were separated, the components were given their own P numbers just as with the other species.

About 900 cultures, representative of those reported in Perkins and Turner (1988) and the recently collected cultures first reported here, were deposited in FGSC and given FGSC accession numbers. They are listed in the printed FGSC catalog (Fungal Genetics Stock Center, 2000), which is also on-line at http://www.fgsc.net. When available, two cultures of opposite mating type were deposited for each species from each geographical area (although not necessarily from each individual site). Whereas most deposited cultures appear to be pure strains, only a minority have been purified by conidial isolation.

Cultures in the FGSC catalog were not given a species label unless the designation was virtually certain. Our standard for species identification for deposited strains was more rigorous than the standard used in Tables 2 and 3 for purposes of presenting an overall picture of the collection. Initially we required that 90% or more of the ascospores were black when a culture was crossed either to the standard species-tester or to another culture serving as an alternate tester because it had been firmly identified in a cross to the standard tester. As will be explained below in the section on species indentification, the required level of black ascospores was sometimes lowered to 50%, especially when other criteria were fulfilled.

The entire Perkins collection, including cultures not listed in the FGSC catalog, is now available from FGSC, together with a complete database.

**Accessing Cultures Not Included in the FGSC Catalog**

The cultures listed in the FGSC catalog show clearly the breadth of the collection and should serve the needs of
most laboratories, but they represent only about 20% of the total. The other 80% of the collection—consisting of additional samples from listed sites and from nearby sites—is now available from FGSC for researchers who wish to study large contiguous populations. These cultures are listed at the FGSC web site as a separate collection but will not be given FGSC numbers or listed in the printed catalog. All the cultures from our collection that are listed in the FGSC catalog have numbers prefaced by the letter P, and this "P number" can be used to identify them in the database for retrieval of detailed information.

The database of the full collection can be used to obtain more extensive data on wild cultures whether or not they are in the FGSC catalog. The 80% of the collection not listed in the catalog includes well-identified cultures from the same vicinity and of the same species as the cultures that are listed. It also includes other cultures that are mixtures or that have less reliable species designation, e.g., those listed as "N. intermedia?" and "N. discreta-like." Specific information on each culture, including results of crosses to species-testers, is included in the database. The database should be consulted before selecting cultures for laboratory study. We reiterate the need to obtain conidial isolates.

In the database, the file containing specific information about each culture is called WILD. In it, the field "source" lists whether the collector was "DDP" or "notDDP" and whether the substrate was "burned" or "notburned." The file in the database named SiteColDat contains site collection data. Name of collector, date, and specific description of the location and substrate are given.

When components of mixed cultures were isolated and given their own P numbers, the original silica gels were retained in the collection. In the database, the records for mixed cultures of all kinds include a listing of any progeny or conidial isolates obtained from them in the field "DATA." The records of the derived strains identify the culture from which they came. Other mixtures were not resolved. The majority of cultures that were mixtures of both mating types of the same species were not separated because we already had both single-mating-type strains of that species from the same site. Seven cultures that included strains of two different Neurospora species were not separated because they appear to repeat the species and mating types of cultures from the same sites.

**Determining the Species of New Isolates**

Although conidiating cultures can confidently be identified as Neurospora on the basis of vegetative morphology and pigment, the species of a strain cannot be determined reliably by vegetative morphology or by the size and shape of ascospores. The fertility of crosses to standard species-tester strains provides a much more reliable criterion. Previous descriptions of our mating-based methods to determine species (Perkins and Turner, 1988; Perkins et al., 1976) were designed primarily for the general reader and are not sufficiently detailed to serve as a guide to future investigators to resolve some of the problems encountered in practice. A more detailed description of our current procedures is therefore given here, including corrections and recent changes.

Crosses were used for species identification. Species-tester strains (Table 1) were normally used as female parents on agar slants of synthetic cross medium (SC). Initial tests with N. crassa fluffy testers were made in 10 × 75-mm culture tubes, which were ready for fertilization after 4 days of incubation at 25°C. The fluffy (fl) mutation confers two advantages, high fertility and absence of macroconidia, which facilitates examination of ascospores shot to the wall of the tube. Furthermore, results of crossing to fluffy testers not only give positive identification of N. crassa cultures but also differentiate between N. intermedia and the remaining species, and they often provide information about mating type, even when the crossing reactions are minimal.

Methods were developed for use of species-testers other than N. crassa. Most of these testers produce conidia, which interfere with the observation of perithecia and ascospores when small tubes are used. Crosses are therefore made in larger, 13 × 100-mm tubes. At 25°C, these cross-tubes are ready for fertilization 5 days after inoculation. Slants (or plates) that are ready for fertilization can be stored at 5°C for 2 weeks without significant loss of fertility. During most of the study, the SC was supplemented with 1 or 2% sucrose, but we now recommend use of filter paper rather than sucrose in tests made with conidiating testers. The filter paper is cut into strips and inserted into the tubed agar medium prior to the autoclaving and slanting processes. When used on filter paper medium, testers for the other species acquire the advantageous qualities just described for the N. crassa fluffy testers. Conidiation is reduced, making perithecia and ascospores easier to see. Fertility is also improved in many cases. Some N. discreta strains are poorly fertile as female parents on SC with sucrose but are fully fertile when filter paper is substituted for the sucrose (Perkins and Raju, 1986). More recently, some wild isolates of N. sitophila have also been shown to require filter paper for successful crossing (see section on N. sitophila). A few strains cross better on sucrose than on filter paper; so, for strains that
are not crossing well to any species-testers on the medium with filter paper, medium with sucrose should also be tried. Generally, assignment of a new strain to a species was based on the obtaining of a majority of viable ascospores in a fertile cross to one of the strains designated as standard testers for that species. Black ascospores are usually viable, brown (slightly less pigmented) ascospores are sometimes viable, and white (unpigmented) ascospores are inviable.
Initially, the criterion of species identity was a fertile cross that gave 90% or more black ascospores with a particular tester. Experience has led us to reduce the required percentage of black ascospores from 90 to 50%. Species assignments based on the weaker requirement have not been overturned by further analysis and have often been supported by follow-up crosses to other strains of the same species. The less rigorous value also makes sense in view of the many factors that are known to reduce the frequency of viable, black ascospores below 90% in crosses between strains of the same species.

When a collected strain made a productive cross with one of the standard species-testers (Table 1A) but did not produce a preponderance of viable ascospores, temporary tester strains that had crossed well to the species-tester were found among cultures from the same vicinity. Some of these alternate testers (Table 1B) were used for cultures from a broad region. Cultures that were self-fertile and produced four-spored asci were classified as *N. tetrasperma* without crossing them to a *N. tetrasperma* species-tester.

Although the great majority of *Neurospora* cultures were easily assigned to a species, there were important exceptions. Most of these are described below in the sections that concern individual species. In addition, some collected cultures were “mixed,” being composed of two or more strains of the same *Neurospora* species or (less often) of different *Neurospora* species. Many of the mixed cultures were resolved into presumably pure strains by conidial isolation.

**N. crassa** and **N. intermedia.** The only described species that are similar enough in crossing behavior to be confused with each other are *N. crassa* and *N. intermedia*. Unlike other interspecific crosses, these species produce large normal-looking perithecia when crossed with each other. Cultures received in the laboratory were first crossed to the *N. crassa* standard fluffy testers, because these testers usually make at least a rudimentary response to most of the other species. Such a response allowed us to determine the mating type. The cultures whose crosses to the *N. crassa* testers produced large normal-looking perithecia and mostly viable (full-size, black) ascospores were classified as *N. crassa*. Almost all *N. crassa* from the Western Hemisphere were quickly and easily identified by this cross to standard *N. crassa* fl testers, which are descendants of the original B. O. Dodge stocks, which originated in the United States (Perkins et al., 1976). In contrast, we observed early in the study that many (but not all) *N. crassa* cultures from India produced more brown than black ascospores in crosses to the testers from the United States. (Even though some brown ascospores are viable, their percentage germination is far lower than that for black ascospores). The selection of India-background testers helped improve the crossing results for *N. crassa* cultures from India and from Thailand and Malaysia.

Unfortunately, the India-background testers were later found to carry a senescence plasmid (Court et al., 1991). Stocks can be maintained on silica gel, however, and they are still available from FGSC. Nonsenescent cultures collected at the same site as that of the original India-background testers are available from FGSC, but they have not been tested extensively enough to warrant their designation here as species-testers.

In practice, a tentative identification of *N. intermedia* cultures based on the initial cross with *N. crassa* testers was also made. Many *N. intermedia* cultures make fertile perithecia with *N. crassa* but produce few viable ascospores. Usually ascospores were abundant, but either none or <10% were black and viable, and the rest were defective. Some *N. intermedia* cultures make few or no ascospores of any type in crosses to *N. crassa*, but the perithecia are large and dark, unlike perithecia from other interspecific crosses.

Putative *N. intermedia* cultures were next crossed to the appropriate *N. intermedia* standard tester and definitively identified by their production of a high percentage of viable ascospores. The original *N. intermedia* testers from Taiwan (FGSC 1766, P13 A and FGSC 1767, P17 a) worked very well with cultures of *N. intermedia* from much of the Eastern Hemisphere but produced few ascospores when crossed with cultures from the United States. Shew (1978) selected the present standard testers (FGSC 3416 and 3417) from descendants of FGSC 1766 × 1767, and these cross reasonably well with most *N. intermedia* collections, including those from the Western Hemisphere.

Most *N. intermedia* cultures from a given area have similar fertility patterns when crossed to the standard *N. intermedia* testers. Some cultures of *N. intermedia* that made few or defective ascospores when crossed to the *N. intermedia* standard testers crossed well and gave more than 50% viable ascospores when crossed to one or another of the firmly identified *N. intermedia* strains from their region. Test crosses of *N. intermedia* cultures from Africa, for example, made abundant pigmented ascospores, of which more than 50% were generally black. But these cultures had varying proportions of ascospores that were dark brown, with low germination, and lighter brown, with no germination, in response to our standard 30-min, 60°C heat shock. Results similar to these were...
sometimes obtained when strains from the same site were intercrossed. Changed culture conditions sometimes resulted in changed proportions of light-colored ascospores. African N. intermedia cultures consistently made abundant white ascospores in crosses to N. crassa. On the basis of these and other observations, we believe that cultures from Africa can safely be designated N. intermedia if a substantial majority of the ascospores in crosses with the standard N. intermedia testers are black, dark brown, or light brown, even if the percentage of black spores falls below 50%. Because ascospore-maturation defects similar to these are seen in intercrosses between the strains being tested, it seems unlikely that the defects reflect partial differentiation into subspecies or species.

Some probable N. intermedia strains from scattered locations made virtually no viable ascospores with N. crassa but made between 10 and 30% viable ascospores with at least one tester of N. intermedia. Often, the crossing results for a particular site or cluster of sites indicated that some of the samples were just over the 50% viability cutoff that we used for FGSC deposits and others were somewhat lower. It is reasonable to assume that all of these cultures are N. intermedia strains with ascospore development problems. These strains were not given FGSC numbers or listed in the regular FGSC catalog, but they are included as N. intermedia in the complete Perkins collection and are tallied as such in Tables 2 and 3. Their crossing results are included in the database of the full collection.

In addition to some of the strains of the yellow ecotype described below, 17 cultures were considered possible N. intermedia even though that diagnosis was not supported by the production of viable ascospores. In part, this designation is based on the predominance of identified N. intermedia strains at the site where they were collected and at other sites in the vicinity. These 17 cultures had no more than rudimentary reactions in crosses with other species, and at the least they made large perithecia with either N. crassa or N. intermedia. No further crossing was observed when they were tested with all new putative species-testers and among themselves. The most likely explanation is that the 17 strains have various dominant reproductive defects. They are listed in the data for the extended Perkins collection as “N. intermedia?” and are tallied in the tables as N. intermedia.

The yellow ecotype of N. intermedia. All of the N. intermedia strains discussed above are orange and are indistinguishable in appearance from typical N. crassa and N. staphila. However, a second ecotype of N. intermedia exists that is saffron yellow, has larger conidia with many more nuclei, and makes more rounded ascospores (Turner, 1987). This yellow ecotype was found almost exclusively on nonburned substrates, most commonly on discarded maize cobs. It has been found only in the Eastern Hemisphere except for one strain from Hawaii. In contrast, the common orange ecotype was usually, but not exclusively, found on burned substrates and was common in both Eastern and Western Hemispheres.

Altogether, 178 cultures of the yellow ecotype have been found, of which 23 were of mixed mating type. Conidial size was measured at 1000× or 2000× magnification for 125 yellow cultures and was observed at 60× for the rest. Conidia were also measured for 158 orange N. intermedia strains from countries where yellow strains had been found. Conidial size was completely concordant with color.

Strains of the yellow ecotype typically show poor fertility when intercrossed under standard crossing conditions on agar synthetic cross medium (Turner, 1987), but the same strains are highly fertile on pieces of maize cob (Pandit, 2000).

The yellow strains were judged to be conspecific with orange N. intermedia because (1) some crosses of yellow strains by standard orange N. intermedia make abundant ascospores with up to 90% viability; (2) although many other crosses involving yellow strains show poor fertility under standard crossing conditions, production of viable ascospores is not significantly better in crosses between two yellow strains than in crosses of yellow by orange; and (3) yellow strains usually behave like orange N. intermedia when crossed to N. crassa: only 1 to 5% of the ascospores are viable. The two ecotypes differ at multiple loci which are not tightly linked (Turner, 1987).

Identification of species should not be based solely on color. Some strains of N. discreta are yellow. Also, yellow mutants have been found in laboratory strains of N. crassa and they could presumably occur in any conidiating species. The color of the yellow ecotype of N. intermedia is not exactly the same as that of these others, but discrimination requires both experience and availability of representative other strains for comparison.

Possible hybrids between N. crassa and N. intermedia. The possibility must be considered that hybrids occur in the field, especially in the case of N. crassa and N. intermedia, from which hybrids are easily obtained in the laboratory. Dozens of cultures were flagged as possible hybrids, but few are still considered candidates for that designation. There were areas where some N. intermedia cultures or N. crassa cultures (as defined by crosses to species-testers other than the standards) made crosses...
with <50% black ascospores when crossed to a standard tester. Usually this relative infertility occurred only with one species but not with the other. However, as previously reported (Perkins and Turner, 1988), some strains, particularly those from the Malayan Peninsula, produced from 10 to 50% viable ascospores in crosses to both the N. crassa and the N. intermedia standard testers. We used crosses with clearly identified local strains to identify the species to which the ambiguous strains belong. Although the results did not support a diagnosis of hybridity, interesting questions remain. For example, a few of the N. intermedia cultures from Andhra Pradesh were fertile and made more than 10% viable ascospores with the N. crassa testers, whereas most of the other N. intermedia cultures from southern India made only large, black, sterile perithecia with the N. crassa testers.

Some cultures, from scattered locations, made normal-looking perithecia but few ascospores, or only a low percentage of viable ascospores, in crosses to both the N. crassa and the N. intermedia testers. This result was similar to those for many laboratory hybrids made between these two species. Nevertheless, we were able to assign most of these problem cultures to one of the species after further study, which usually included conidial isolation and crosses to indented strains from the same region. The strains that could not be assigned to either species were deposited in FGSC as “Possible hybrids between N. crassa and N. intermedia.” They are not included in Table 2 but are listed individually in Table 4. Those collected after 1988 are listed in both Table 3 and Table 4.

N. sitophila. Species identification of N. sitophila requires the careful use of studied testers. Test crosses should be made with crossing medium that uses filter paper as the carbon source instead of sucrose. Many of the N. sitophila strains, from culture collections or from nature, that we attempted to use as female parents at an early stage of the collection project were female sterile or produced mostly defective ascospores. We still urge caution in interpreting poor results from crossing two randomly selected N. sitophila strains. However, our early results might have been different had we used filter paper as a carbon source instead of sucrose.

Perkins and Turner (1988) used filter paper as the carbon source only when crossing putative N. discreta strains, and its efficacy was attributed to the greater fertility of the female parent on the medium with paper. More recently, we observed that some strains of N. sitophila will act as a male parent on crossing medium containing only filter paper as the carbon source but are completely sterile on medium containing sucrose, even when used to fertilize female parents that have produced abundant protoperithecia (Fairfield and Turner, 1993).

About 10% of the N. sitophila cultures in the collection at the time of this discovery had not been identified because they did not cross with the N. sitophila testers on medium with sucrose. Examples are FGSC 6853 (P4090 A) from Rarotonga, FGSC 6863 (P4376 a) from Ivory Coast, and FGSC 6850, which is described in the following paragraph. Initially this subset of strains was reported as a putative new species called N. celata (Turner and Fairfield, 1990) and they were included as such in 1990 and 1992 in the FGSC Catalog of Strains.

A spore killer element, Sk-1, was found in many of the collected N. sitophila strains (Turner and Perkins, 1979, 1991; Turner, 2001). Ascospore maturation is normal in homozygous crosses (in which both parents carry the Sk-1 killer element or both are sensitive to it), but in a cross in which this element is heterozygous, half of the ascospores in each ascus are shrunken and colorless (4:4 ascii). With experience, the mixture of black and aborted spores can be observed on the wall of the cross-tube. Researchers working with this system for the first time should open perithecia to determine which crosses are making 4:4 ascii and then observe the appearance of the cross-tubes. Fertile N. sitophila species-testers were developed before Sk-1 was recognized. These were later found to be carrying Sk-1. Fertile testers that are sensitive to killing by Sk-1 were then developed. These sensitive strains, like the original Sk-1 testers, can be used to screen N. sitophila from all regions. Some putative N. sitophila cultures were crossed first to Sk-1 testers and others were crossed first to testers that are sensitive to Sk-1. If the resulting asci were 4:4, the Sk-1 phenotype of the new culture was clearly the opposite of the tester, and the aborted ascospores did not affect the species designation. All N. sitophila cultures were characterized for their killer phenotype, and only one, FGSC 6850 (P4601 from Gabon), made ascii with all black ascospores regardless of the Sk-1 genotype of the other parent, indicating that P4601 carries a gene or element that confers resistance to killing by Sk-1 but does not carry the gene or element that results in killing when crossed to Sk-1-sensitive strains.

N. tetrasperma. Most but not all of the N. tetrasperma cultures isolated in the field are self-fertile and produce perithecia in the original culture tubes on minimal medium. Confirmation is obtained for the self-fertile cultures by observation of four-spored ascii. Most of the strains deposited as N. tetrasperma have been identified only by this criterion. Of over 400 collected cultures of N. tetrasperma, only 8 (2%) were of single mating type. These
were identified by being crossed to the homokaryotic N. tetrasperma A and a testers. The single-mating-type isolates of N. tetrasperma are identified in crosses to one of the N. tetrasperma species-testers.

Whether asci are four-spored or eight-spored can usually be determined by examination of the wall of the cross-tube soon after ascospores are shot from the perithecia, before the wall becomes crowded with spores. Opening of the perithecia is usually not necessary. Regardless of whether four-spored asci were observed in the original culture tube, all newly collected, self-fertile, putative N. tetrasperma cultures were crossed to the N. crassa testers to detect possible mixtures with another species. When cultures with N. tetrasperma also contained N. crassa or N. intermedia, the cross resulted in sectors of both eight-spored and four-spored asci, and the separate components could be purified. A trace contamination of N. tetrasperma by N. sitophila or N. discreta would not be revealed by our standard procedure. We have observed examples of such mixtures, however, because the other species overgrew the sparsely conidiating N. tetrasperma component after several serial transfers of conidia.

Not all strains of N. tetrasperma are interfertile (Jacobson, 1995). As with the heterothallic species, additional N. tetrasperma testers may be required in the unlikely event that unknown cultures do not cross to any of the standard testers. Generally, the closer the geographic origin of two collected N. tetrasperma strains, the more successful the cross; this result provides one criterion for the selection of additional testers.

**N. discreta.** For N. discreta, we had to modify our approach to species identification. This species (or perhaps species cluster) was especially difficult to handle because of its more exacting crossing requirements and because of as yet unidentified factors that result in high levels of ascospore inviability even from crosses between closely related strains.

Because some N. discreta strains and some N. sitophila strains cross poorly on sucrose, tests of unknowns that do not react to N. crassa should be made on synthetic crossing medium with filter paper substituted for the sugar (Fairfield and Turner, 1993). Examples of N. discreta strains showing poor female fertility on sucrose are Homestead-1k a (P390; FGSC 3268) and Santa Maria a (P755; FGSC 3319).

A simple observation of percentage and abundance of viable ascospores from crosses with standard testers, such as we use to distinguish N. crassa from N. intermedia, cannot be used with N. discreta. Strains that have been selected as good female parents usually either lose fertility or begin producing defective ascospores in pairings that previously produced viable ascospores. Preservation by freezing at −20°C or by dehydration on silica gel does not prevent this deterioration. Progeny pairs from a cross that gave mostly viable ascospores often produce mostly defective ascospores, as do some back crosses to the parents.

We classified as “N. discreta-like” those cultures that crossed to no other species-testers but that produced a low percentage of black ascospores when crossed with the N. discreta testers. For purposes of reporting, we have combined them with designated N. discreta cultures as the “N. discreta group.” Additional isolates were designated as N. discreta-like because, though they produced no viable ascospores with identified N. discreta strains, they did produce at least some black ascospores with strains that were already placed in the N. discreta-like group. This method has resulted in a set of designated cultures concentrated mainly in one region, the Ivory Coast. This result supports the assumption that there is an evolutionary relationship among these cultures. The crossing problems in the N. discreta group may be related to a few genes for ascospore production rather than to species divergence. With the exception of a small group of strains presented in the following paragraph, we did not find that the strains designated as N. discreta-like intercross with one another and produce a high percentage of black ascospores. Therefore, we do not think that the N. discreta and N. discreta-like cultures are a closely related pair of distinct species such as N. crassa and N. intermedia.

A group of isolates with crossing behavior that suggests that they are a putative new species most closely related to N. discreta. Seven isolates from the Ivory Coast were highly fertile and produced over 90% viable ascospores in crosses among themselves. They produced no more than 10% black ascospores when crossed with the N. discreta species-testers and produced no black ascospores when crossed with testers representing the other known species. However, these isolates made perithecia when crossed to the N. intermedia testers, and in three cases the perithecia produced white ascospores. FGSC Accession Nos. of the strains are 8318 and 8334 through 8339. We suggest the use of P4527 A (FGSC 8338) and P3660 a (FGSC 8318) as reference testers for this group of isolates.

### RESULTS

#### The Collection

The cultures summarized in Table 2 came from 735 collection sites representing a worldwide sampling of the...
### TABLE 2
Wild-Collected Conidiating Neurospora Cultures That Have Been Assigned to Species Since 1968, Listed by Region of Origin

<table>
<thead>
<tr>
<th>Region</th>
<th>All species</th>
<th>N. crassa</th>
<th>N. discreta</th>
<th>N. intermedia</th>
<th>N. sitophila</th>
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<td>143</td>
<td>3020</td>
<td>568</td>
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Note. This table lists all strains that can be placed, even provisionally, in a single species category, including those published previously and most of those in Table 3. Possible hybrid strains are listed separately in Table 4. Unresolved mixed cultures of two mating types of the same species are listed here as one culture. No cultures of mixed species are included here. Any of the cultures collected as mixed species were separated into pure strains, which are included here, but separating cultures is not part of the standard protocol. When assurance of purity is important, cultures of interest should be purified as a precaution. Seven additional mixed cultures (from five different geographic areas) were not resolved and are not included here.

Tropics and subtropics. Table 2 includes both newly collected cultures, described in the next paragraph, and new information about some of those listed previously by Perkins et al. (1976) and Perkins and Turner (1988). The 202 cultures listed without species identification in Perkins and Turner (1988) were subjected to additional analysis. This
analysis usually included the crossing to many different testers and the plating of conidia, which sometimes yielded several different strains. Definite or tentative assignment was then made to a species. From the earliest to the most recent collections, some samples contained more than one Neurospora species. Except for 7 cultures, all mixed-species cultures have been processed and are now included as one, two, or three derived strains. Tables 2 and 3 tally the components of all processed mixtures but not the original mixed cultures. Single-species mixtures that were not separated are counted as 1 culture. Cultures of different mating types of a single species were not necessarily separated if both components of the mixed cultures were the same as other samples at the site. Nevertheless, mixed cultures of different mating types of a single species were not necessarily separated if both components of the mixed culture were the same as other samples at the site.

Seventy-eight sites comprising 611 cultures have been added to the collection since the 1988 report. These additional sites have broadened the geographic range and increased the environmental diversity of the collection. Most of the 3900 strains reported in 1988 were collected systematically by D. D. Perkins. When possible, 7 to 10 discrete colonies were sampled from each burned site. The cultures added since 1988 have been contributed by many individuals who have used a variety of methods. Sometimes only a single culture was obtained from a site. Table 3 lists the new cultures by region and collector. New countries or islands that were sampled include Cayman Islands, Dominican Republic, Trinidad, French Guiana, Madagascar, Swaziland, and Rarotonga. Many of the new sites were from previously unsampled regions and were represented by only a few cultures, so it was important to make a thorough analysis of each sample and to extract multiple strains if they existed. Nevertheless, mixed cultures of different mating types of a single species were not necessarily separated if both components of the mixed culture were the same as other samples at the site.

Previously, Mexico was represented by only 5 samples from the state of Baja Sur in the West. The 75 new samples are from the eastern states of Yucatan and Quintana Roo, in the Yucatan Peninsula. In his collecting there, R. L. Metzenberg included soil samples that yielded mixed cultures of conidiating Neurospora species. We put these cultures through successive separations to obtain 33 pure strains, which were given P numbers and added to the collection. The array of species among these soil samples paralleled the species that were obtained at the same time from burned vegetation.

From both the old and the new collections there now remain 11 cultures that are possible hybrids between N. intermedia and N. crassa (Table 4). Nine of these putative hybrids have been deposited in FGSC.

**Distribution of Species**

The genus is ubiquitous in humid tropical and subtropical regions, but populations differ from region to region with regard to which species are present (Fig. 1, Table 2). This diversity does not result in complete geographic separation of any two species. Indeed, all five species were found at one site in the Yucatan. Elsewhere, there were five sites at which four species were found and many other sites at which three species were found.

N. crassa was found regularly at burned sites in Africa, in India, and in the Western Hemisphere, where it is concentrated in and near the Caribbean and extends to northern Brazil and the southeast corner of the United States. N. intermedia, by far the most common species, was found in the entire range of N. crassa and in southern Brazil, across southern Asia, in Australia, and on islands all across the Pacific. In fact, N. intermedia was found in moist tropical and subtropical environments at all latitudes sampled. N. discreta was found as a major component of collected species mainly in the Ivory Coast. Individual cultures of N. sitophila and N. tetrasperma were abundant in Tahiti, the Americas, and West Africa, but individual cultures of these species were collected from burned vegetation in many other regions. N. tetrasperma was the predominant species (72% of all samples) collected in New Zealand, which is the southernmost area sampled. On the other side of the equator, N. tetrasperma was found with increasing frequency as collecting moved northward in the United States. Ito (1988) recovered N. tetrasperma from bonfire sites in Japan. Thus, there is at least a suggestion that N. tetrasperma may tolerate a greater range of temperature and moisture than the other species.

**Neurospora from Nonburned Substrates**

The original design of the project was to concentrate on samples from burned vegetation. Perkins and Turner (1988) listed samples from nonburned substrates separately from the samples from burned substrates. Cultures collected since then cannot be listed separately for burned vs nonburned because some of the collectors did not provide information on substrates. Most individual colonies growing on vegetation after a fire appear to have originated from different ascospores (Perkins et al., 1976), and they appear to represent a local breeding population. On the other hand, colonies on discarded food or on sugar factory waste may be clonal in origin, following long-term vegetative propagation. Our experience has been that col-
<table>
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<td></td>
<td></td>
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<td></td>
<td>N. intermedia</td>
<td>Norman Giles</td>
<td>5</td>
</tr>
</tbody>
</table>
lections from such sites tend to be less varied in species, morphology, and mating type than collections from burned sites, yet many nonburned sites yield several phenotypes, suggesting that multiple samples are worth being collected.

The natural history of contaminants found indoors in climates hostile to Neurospora presents an unresolved problem. Colonies growing indoors, especially in regions where Neurospora is not found in nature, could represent direct conidial contamination from imported foodstuffs, but this explanation was not supported by a study of Neurospora in bakeries by Yassin and Wheals (1992). Samples of contaminants from 18 sites have been sent to us from laboratories, offices, and kitchens in scattered regions. Most of them are N. sitophila, and they come from areas, such as New York state, that are well north of the zone where Neurospora is found outdoors and even farther from the latitudes where N. sitophila is abundant on burned vegetation.

Samples from Australia offered an interesting set of substrates. Shaw (1990a, 1998) has documented individually all Neurospora cultures collected in Australia. (See also Table 3. Some of these cultures were also included in the summary tables of Perkins and Turner, 1988). One N. sitophila strain and 18 N. intermedia strains were obtained from the pollen-collecting baskets of bees in conjunction with Shaw’s ongoing study (Shaw and Robertson, 1980; Shaw, 1990a,b, 1993, 1998, and personal communication). Conidia harvested by the bees came from colonies on filter mud from sugar refineries (Fig. 2) or from steamed logs. J. Tierney and G. Hughes (Table 3) collected Neurospora from lumber or logs that had been steamed.

There have been numerous other reports of Neurospora from nonburned substrates. In New Zealand, G. I. Robertson (Table 3) found N. intermedia growing on soil that had been steamed for use in a greenhouse, and N. intermedia perithecia were found on logs exported from New Zealand (Ridley, 1994). J. Leslie (Table 3) collected 1 N. intermedia and 13 N. sitophila cultures from bags of steamed sawdust used for mushroom cultivation in Thailand. Neurospora has also been reported from maize silage (Albert and Krawczyk, 1992). In Brazil, Park et al. (1982) and Pastore et al. (1944) recovered Neurospora cultures.

### Table 3—Continued

<table>
<thead>
<tr>
<th>Region of origin</th>
<th>Species</th>
<th>Collector</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>U.S.A. (Continental)</td>
<td>N. crassa</td>
<td>Jeff Hoy</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>David Jacobson</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>N. intermedia</td>
<td>Jeff Hoy</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>N. sitophila</td>
<td>Anonymous</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>N. tetrasperma</td>
<td>Jeff Hoy</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>David Jacobson</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Robert Metzenberg</td>
<td>1</td>
</tr>
<tr>
<td>Venezuela</td>
<td>N. crassa</td>
<td>Norman Giles</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>N. tetrasperma</td>
<td>Norman Giles</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>601</td>
</tr>
</tbody>
</table>

Note. The cultures listed are included in the summaries of Table 2, with the following exceptions:

- Appear to be a mixture of two strains that repeat species and mating type of other strains from the same site. They are among the seven cultures mentioned in the note to Table 2.
- These five possible hybrid strains are listed also in Table 4, but not in Table 2.

### Table 4

Possible Hybrids of N. crassa and N. intermedia

<table>
<thead>
<tr>
<th>Site</th>
<th>Region</th>
<th>Isolation No. and mating type</th>
<th>FGSC No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carrefour Dufort</td>
<td>Haiti</td>
<td>P3426A</td>
<td>8225</td>
</tr>
<tr>
<td>Carrefour Dufort</td>
<td>Haiti</td>
<td>P3425A</td>
<td>8200</td>
</tr>
<tr>
<td>M allilintham</td>
<td>India</td>
<td>P4335 a</td>
<td>8203</td>
</tr>
<tr>
<td>M adurai</td>
<td>India</td>
<td>P2543 a</td>
<td>8198</td>
</tr>
<tr>
<td>Georgetown</td>
<td>Malaysia (Penang)</td>
<td>P2632A</td>
<td>8199</td>
</tr>
<tr>
<td>M erida-1</td>
<td>Mexico</td>
<td>P4158 a</td>
<td>8202</td>
</tr>
<tr>
<td>M erida-1</td>
<td>Mexico</td>
<td>P4157 a</td>
<td>8201</td>
</tr>
<tr>
<td>M erida-2</td>
<td>Mexico</td>
<td>P4145 a</td>
<td></td>
</tr>
<tr>
<td>Nosy Be</td>
<td>Madagascar</td>
<td>P4578 a</td>
<td>7848</td>
</tr>
<tr>
<td>Nosy Be</td>
<td>Madagascar</td>
<td>P4576A</td>
<td>7847</td>
</tr>
</tbody>
</table>

Note. These strains are not included in Table 2. Both N. crassa and N. intermedia were collected in the vicinities of the first eight cultures. The two from Madagascar, which are the only available strains from that island, produce 95% black ascospores when crossed to each other. They were used as parents with six of the others, which were also intercrossed, but none of the pairs appeared to be conspecific. The M erida-2 strain has been lost.
obtained from beiju, a fermented cassava product used in the state of Maranhão to make an indigenous liquor. These strains, which we identified as *N. sitophila* (Turner, 1992), are fragrant because they produce ethyl hexanoate, which has a fruity odor. Samples of *Neurospora* from bakeries in England were analyzed over an extended period by Yassin and Wheals (1992). *N. crassa* was the predominant species, with *N. intermedia* and *N. sitophila* also present. These authors concluded that the contamination was not endemic to the bakeries and was not brought in by flour or other ingredients. They suggested that perhaps new cultures arrived on returned baked goods. This conclusion leaves unresolved the identity of the original source of contamination.

**DISCUSSION**

**Advances in Knowledge of Species and Populations**

Work in our own laboratory has concerned the delineation of species, their natural history and geographical distribution, the incidence of meiotic drive elements and strains resistant to drive, and the significance of pseudohomothallism as exemplified by *N. tetrasperma*.

**Species relationships.** Early in the study, we chose to use mating-based criteria to assign isolates to species. Recent experience and the use of molecular criteria of relationships (e.g., Skupski et al., 1997) have reinforced our confidence in the method. The choice was made for both theoretical and practical reasons. Mating capability is of central importance if we think of each sexual species as a reproductive community that shares the same gene pool (see Perkins, 1991). Crossing ability is also the most convenient and often the only practical way to make species assignments in a genus in which morphological differences between individuals of different species are tenuous, and intraspecific variations in ascospore and vegetative morphology may be as great as those between species. Perkins (1994a) made recommendations for avoiding ambiguity when describing and categorizing the mating behavior of interspecific pairs.

DNA sequence comparisons now provide criteria to determine phylogenetic relationships that are independent of mating ability and the productivity of crosses. (See, for example, Taylor et al., 1993). Molecular data on relationships of *Neurospora* species were first obtained using RFLPs in random fragments of nuclear DNA (Natvig et al., 1987) and length mutations in mitochondrial DNA (Taylor et al., 1986; Taylor and Natvig, 1989). The polymerase chain reaction made greater resolution attainable.
using DNA sequence comparisons for various cloned genes and regions (Metzenberg and Randall, 1995; Randall and Metzenberg, 1995, 1988; Skupski et al., 1997). The various analyses agree in showing distinct differences between the established species and in showing that N. discreta is the most distant from the other species. For species other than N. discreta, a tree based on mating type A-1 differs in several respects from trees based on sequences elsewhere in the genome (Fig. 2 in Skupski et al., 1997; Metzenberg and Randall 1994; discussed below under Mating type). The inconsistencies between trees based on various molecular sequences may reflect polymorphisms that predate speciation, or they could be the result of horizontal transfer of chromosomes or chromosome segments between already established species.

**Geographical distribution, dispersal, and ecology.** Most samples were taken and field observations made during brief visits, often incidental to travel for other

**FIG. 2.** Neurospora growing on sugar refinery filter mud spread as fertilizer on fields near Cairns, Australia (top); a close-up view (bottom). Honeybees fill their pollen baskets with conidia from colonies such as these (Shaw and Robertson, 1980; Shaw, 1998). Photographed by R. E. Beever.
purposes. Many geographical regions have not been ade-
quately sampled. Despite these shortcomings, it seems
clear that different conidiating Neurospora species main-
tain different geographic distributions, although no two
species have completely separate ranges.

As with pelagic marine organisms, the principle of com-
petitive exclusion (Hardin, 1960), which states that two
species with identical ecological requirements cannot oc-
cupy the same ecological niche in the same territory,
seems not to hold for Neurospora species. All five conid-
iating species were found in abundance in some geographic
regions, notably Haiti and Ivory Coast (Table 2). In re-
gions of abundance, two or more Neurospora species were
often found at the same burned site. As many as four
species were represented in samples at single sites in
Haiti, Ivory Coast, and the Malay Peninsula, and all five
were present at a single site in Mexico.

Pandit and Maheshwari (1994, 1996a,b) studied N. in-
termedia from sugar cane fields where the cane is grown
perennially and subjected to periodic burning. The results
of their field observations and experiments bear on the
activation of ascospores by heat and chemicals, the roles of
conidia and ascospores in dispersal and survival in the soil,
the sexual phase and conditions favoring its occurrence in
nature, and the occurrence and behavior of heterokaryons
under natural conditions. Their failure to recover Neuro-
spora ascospores in repeated air samples at the study site
casts doubt on whether airborne ascospores play a signif-
ificant role in dispersal. Except for their work, no sustained
ecological study of a local Neurospora population has been
undertaken in a geographical area where the organism is
indigenous. Whether population structure and reproduc-
tive behavior in a perennially burned sugar cane field is
representative of Neurospora in various other environ-
ments is not known.

The sexual phase has been elusive in field studies. Peri-
thecia have been reported under the bark of fire-injured
trees (Kitazima, 1925; D. O. Natvig, 2000, personal com-
munication; Jacobson et al., 2001), below the epidermal
tissue of scorched sugar cane (Pandit and Maheshwari,
1994, 1996a), and on corn (maize) cobs carrying the yellow
ecotype of N. intermedia (Pandit et al., 2000). Sexual
structures may not have been observed more frequently
because of difficulty in the recognition of black perithecia
on burned substrate, or concealment of perithecia within
the substrate, or delay of sexual reproduction until conidial
blooms have dispersed.

When soil samples collected in the tropics are subjected
to heat and chemical treatment appropriate to activate
ascospores and to kill vegetative cells, homothallic Neuro-
spora strains are recovered, together with representatives
of heterothallic species (see, for example, Glass et al.,
1990). All the known homothallic Neurospora strains are
aconidiate and all of them came from soil samples. Two
homothallic strains, designated N. terricola and N. pan-
nonica, have come from soil samples in temperate regions
outside the usual range of Neurospora. These are both
quite unlike the heterothallic Neurospora species in traits
other than the longitudinal ascospore striations responsi-
ble for their assignment to the genus. Their proper taxo-
nomic status is uncertain. For discordant features of N.
terricola, see Beatty et al. (1994); for N. pannonica, see

R. Maheshwari (personal communication) has pointed
out that the presence of ascospores in soil does not imply
that soil is the natural habitat for growth. Homothallic
strains of Gelasinospora are commonly recovered from soil
samples (Glass et al., 1990), and Gelasinospora perithelia
have been found together with those of N. intermedia on
old scorched sugar cane stumps (Pandit and M. maheshwari,
1994). We do not know the natural substrate on which
the homothallic Neurospora isolates grow and reproduce.
Their ability to use sucrose as a carbon source suggests
that they are not coprophilic, because dung-dwelling as-
comycetes such as Sordaria, Podospora, Sporormiella, and
Ascobolus cannot use sucrose (Asina et al., 1977; Bretzloff,
1954; Lilly and Barnett, 1951; Yu-Sun, 1964). Conceivably,
both homothallic and heterothallic Neurospora species
normally complete their life cycles inconspicuously in
compost, where ascospores could be activated chemically.
We found conidiating Neurospora colonies on scorched
logs and litter in newly cut and burned primary rain forest
near Mt. Kinabalu in Borneo—a habitat not normally
subjected to burning. Perhaps the dramatic conidiating
blooms seen on burned or scorched vegetation are excep-
tional sporadic events punctuating a mode of growth that
is otherwise inconspicuous or invisible.

Conditions that promote crossing of the various species
under field conditions are largely unknown. Differences
in conditions required for crossing in the laboratory may
reflect the habitats in which crossing occurs in nature. In
N. tetrasperma, the sexual cycle is not inhibited by levels
of ammonium nitrogen or by elevated temperatures that
would block crossing of N. crassa or other heterothallic
species (Viswanath-Reddy and Turian, 1975, and our un-
published observations). Strains of N. discreta and N.
sitophila that cross well on filter paper but not when
sucrose or glucose is provided as the carbon source are
known (Perkins and Turner, 1988; Fairchild and Turner,
1993). The yellow ecotype of N. intermedia crosses well on
pieces of corn cob but poorly on standard crossing medium (Pandit et al., 2000).

**Meliotic drive.** Three species in the worldwide collection were surveyed for strains containing Spore killer elements—chromosomal entities which, when crossed to a sensitive strain, result in the death of progeny that do not carry the killer element. A killer symbolized Sk-1 was found widely distributed in N. sitophila. Sk-2 and Sk-3 were found in only a few N. intermedia strains, from Borneo, Java, and Papua New Guinea. Strains that carry genes conferring resistance to killing by Sk-2 or Sk-3 are present mainly in the same geographical area (Turner, 1990, 2001).

**N. tetrasperma as Representative of a Unique Genetic System**

Availability of many recently collected cultures and recognition of its potentialities have led to a resurgence of interest in N. tetrasperma, both in nature and in the laboratory (see Perkins, 1994b). N. tetrasperma and Podospora anserina, with asci that contain four heterokaryotic self-fertile ascospores, are representatives of genetic systems based on heterokaryosis and therefore unique to fungi. In nature, strains of both species are typically heterokaryons with component nuclei of opposite mating type. Programming of ascus development is different in the two genera, suggesting independent origins of the four-spored condition, but the end result is the same in both (see Raju and Perkins, 1994). These four-spored species superficially resemble true homothallic species in that each ascospore can produce a self-fertile culture. Both four-spored species possess mating types similar to those of heterothallic Neurospora species, however, and when the two components of the heterokaryon are separated, the resulting monokaryons behave like sexually compatible strains of a heterothallic species. Podospora workers have emphasized this interaction by calling P. anserina heterothallic. More commonly, the terms “pseudohomothallic” (Dodge, 1957) or “secondarily homothallic” (Whitehouse, 1949) are used for species such as N. tetrasperma and P. anserina.

These fungi appear to enjoy the benefits of both homothallism and heterothallism. Cultures of single ascospore origin can colonize a new substrate or habitat and initiate sexual reproduction without the need to seek a partner. At the same time, monokaryotic A and a derivatives have the potential to outbreed. Raju (1992) has shown that as many as 10% of ascospores and 20% of conidia from nonmutant A + a N. tetrasperma heterokaryons are self-sterile single-mating-type homokaryons capable of being fertilized by compatible nuclei from either homokaryotic or heterokaryotic cultures.

In N. tetrasperma, presence of mat A and mat a nuclei in the same cytoplasm or in the same nucleus does not evoke an incompatibility reaction. In N. crassa, in contrast, mat A and mat a strains are vegetatively incompatible unless the recessive allele tol is present in both components. Jacobson (1992) has shown that the mutant allele tol in N. crassa corresponds to the allele that is present in wild-type N. tetrasperma (tol1). The wild-type tol allele of N. crassa (tol0) makes mat A and mat a strains heterokaryon incompatible when it replaces tol1 after introgression into N. tetrasperma. Conversely, tol1 makes mat A and mat a strains heterokaryon compatible when it replaces tol0 in N. crassa.

Merino et al. (1996) and Jacobson (1995) designed experiments to determine whether N. tetrasperma outcrosses in nature. Heterokaryotic N. tetrasperma cultures from different geographical regions were resolved into their mating type A and a components. Merino et al. (1996) constructed evolutionary trees from RFLP data, using as probes cloned sequences known to map at various loci spread throughout the mating type chromosome and in three other chromosomes (“autosomes”). The tree based on loci in the mating type chromosome is strikingly different from the tree based on autosomal sequences. When RFLPs in the mating type chromosome are used, clustering is by mating type, with A strains from New Zealand closely resembling the A strains from Hawaii in sequence and differing from the A strains from New Zealand that were originally their partners in the same heterokaryon. In contrast, when the tree is based on autosomal loci, the clustering is geographic, with cultures from the same region resembling each other and with A and a derivatives from the same heterokaryon alike or nearly so. These results are consistent with experimental evidence that crossing-over is abolished or greatly reduced in the mating type chromosome but not in other chromosomes (Howe and Haysman, 1966; Gallegos et al., 2000; our unpublished observations). N. tetrasperma appears to be permanently heterozygous or heterokaryotic for the mating type chromosome. In contrast, the other chromosomes of each heterokaryotic culture are mostly homozygous, as expected if there has not been extensive recent outbreeding. Experiments using genetic markers have confirmed that crossing-over is suppressed in much of the mating type chromosome and have revealed that an obligate exchange occurs distal to the suppressed region. The genetic results are paralleled by cytological observations of asyn-
apasis and localized pairing in pachytene bivalents (Gallegos et al., 2000).

When components of the same wild-collected N. tetrasperma heterokaryons were used for outcrosses between mating type A strains from one heterokaryon and mating type A strains from another, most combinations suffered from defects in the sexual phase even though A and a components from each individual heterokaryon were fully fertile when the components were put together again (Jacobson, 1995). Sexual dysfunction shows different degrees of severity with different combinations. These observations provide yet another indication that outbreeding of N. tetrasperma is quite limited in the field.

Whereas individual isolates are homoallelic for het loci, a survey of N. tetrasperma strains from diverse sources revealed the presence of different, functionally incompatible het-c alleles. These are of molecular types that predate the origin of N. tetrasperma (Powell et al., 2000). Outcrossing has apparently been sufficient to maintain het-c allelic diversity.

Randall and Metzenberg (1995, 1998) showed that a variable flanking region distal to the mating type locus in Neurospora may contain DNA sequences that are species specific and mating-type specific. The mat A and mat a components of N. tetrasperma were separated from various wild-collected cultures. For most N. tetrasperma isolates, the variable sequence in the A component was similar to that in N. intermedia strains of mating type A, and the a component was like that in N. intermedia a, but for 2 of 16 N. tetrasperma cultures from different collection sites, the result was dramatically different. In the two exceptions, from Borneo (T 220-7, FGSC No. 7325) and from Mexico (Coba 131, FGSC No. 7585), the variable region in the A component resembles that of N. crassa or N. sitophila rather than that of N. intermedia, whereas the a component is unexceptional. This suggests that the mating type chromosomes of most of the N. tetrasperma strains originated from N. intermedia, but in the two exceptions, the A mating type chromosome originated from N. sitophila or N. crassa rather than from N. intermedia (M. Metzenberg and Randall, 1995, and personal communication). These authors suggest that deleterious mutations gradually accumulate in the permanently heterozygous mating type chromosomes of N. tetrasperma. Then, periodically during evolution, a deteriorated mating type chromosome is replaced following a cross between N. tetrasperma and one of the heterothallic species. This hypothesis is consistent with the observation that different relationships are shown between Neurospora species when an evolutionary tree based on DNA sequences of homologous regions in a mating type gene is compared with trees based on homologous regions elsewhere in the genome (see Skupski et al., 1997).

N. tetrasperma strains from different sources were used to study mitochondrial migration and replacement following fusion of mycelia (Lee and Taylor, 1993). Single-mating-type mat A and mat a isolates were inoculated on opposite sides of a petri dish. Fusion of hyphae occurred when the two growing mycelia met in the middle. Nuclei of one mating type (the donor) then invaded mycelia of the opposite mating type (the acceptor) to reconstitute a heterokaryon. Invasion was unidirectional in any one dish, but either parent could act as acceptor in different dishes. Ten days later, mitochondrial DNA RFLP differences showed that mitochondria of the donor type had been replaced completely by those of the acceptor type throughout the culture.

**Other Laboratory Investigations Using Naturally Occurring Variants**

Our 1988 review cited a long list of publications reporting the use of variants from nature for experiments in the laboratory. Some of these studies defined newly discovered loci. New examples are cdr (Levine and Marzluf, 1989), mcb and mcm (Maheshwari, 1991), and sen (Navaraj et al., 2000). Other studies involved new alleles at already established loci, for example, a novel nmr allele (Young and Marzluf, 1991). Naturally occurring duplicated and nonduplicated versions of a 5S RNA gene were used in a study relating DNA methylation to duplication (Selker and Stevens, 1987). Naturally occurring recessive genes that impair the sexual phase were examined cytologically for their effects on ascus development (Raju and Leslie, 1992). Fatty acid composition was examined in 14 wild isolates representing five Neurospora species (Goodrich-Tanrikulu et al., 1999).

Most of the recent work on natural variants falls into five major categories: mitochondrial plasmids, genes governing vegetative incompatibility, mating type genes, meiotic drive elements (Spore killers), and transposable elements. Progress in each of these areas is summarized in the following sections.

**Mitochondria and mitochondrial plasmids.** Strains from nature have provided material for both evolutionary and experimental studies on mitochondria and mitochondrial plasmids (reviewed by Griffiths et al., 1995; Griffiths, 1995). Length mutations in mitochondrial DNA were used to determine variability within and between species and to infer evolutionary relationships (Taylor and Natvig, 1989).
Surveys of the worldwide collection (Yang and Griffiths, 1993; Arganoza et al., 1994) have revealed plasmids in all five conidiating Neurospora species. Overall, plasmids were found in about half of the strains examined. Numerous plasmid types were found, both linear and circular. More than one type may be present in the same strain. New types may arise by recombination, and plasmids may be lost (Griffiths and Yang, 1995; D ebets et al., 1995). All the known Neurospora plasmids are associated with mitochondria.

Horizontal transfer of plasmids between species, between strains of the same species, and between different marked mitochondrial genotypes has been inferred from the distribution of plasmid types in strains from nature and/or has been demonstrated experimentally using unstable vegetative fusions or transmission from the male through the trichogyne (May and Taylor, 1989; Collins and Saville, 1990; Griffiths et al., 1990; Yang and Griffiths, 1993; M arcinko-Kuehn et al., 1994; Arganoza et al., 1994; D ebets et al., 1994). Sequence divergence of a linear and a circular plasmid from distant geographical locations and in different species and ecotypes is consistent with vertical descent, but horizontal transmission cannot be ruled out (Xu et al., 1999).

Important new findings have been made with two intensively studied small circular mitochondrial plasmids, Mauriceville and Varkud (first reported by Stohl et al., 1982). These plasmids encode reverse transcriptases that resemble plant viral RNA-dependent RNA polymerases and that can initiate cDNA synthesis without using a primer—an unprecedented ability (Wang and Lambowitz, 1993, and references therein; reviewed by Lambowitz and Chiang, 1995). Certain natural isolates with the Varkud plasmid contain a small unrelated mitochondrial RNA, termed Varkud-specific RNA (VS RNA), which performs RNA self-cleavage and ligation reactions. VS RNA is transcribed from VS plasmid DNA by mitochondrial RNA polymerase. It is then reverse-transcribed by the Varkud-specific reverse transcriptase. The VS RNA thus replicates by reverse transcription as a satellite of the Varkud plasmid (K ennell et al., 1994, 1995, and references therein). These observations suggest a relationship of the plasmids to primitive retroelements that were ancestral to retroviruses.

Senescence. Most of the plasmids found in nature appear to have no effect on their hosts, but a few cause senescence (reviewed by Griffiths, 1992, 1998). Local populations and worldwide samples have been examined for the presence of senescent strains (M arcinko-Kuehn et al., 1994; Yang and Griffiths, 1993). Two senescence plasmids have been studied intensively: pKALILO, found in N. intermedia from Hawaii, and pMARANHAR, found in N. crassa from India. Although the two plasmids are nonhomologous at the DNA level, they share features such as inverted terminal repeats. Onset of senescence is preceded by integration of plasmid DNA into the mitochondrial chromosome by a unique mechanism involving generation of long inverted repeats of mitochondrial DNA at the ends of the insert (Bertrand and Griffiths, 1989; Chan et al., 1991; Court et al., 1991). Although plasmids homologous to pKALILO are uncommon, four subtypes are known, with modifications in the long terminal repeats. Three of these subtypes were present in strains belonging to four Neurospora species from Hawaii, Tahiti, Thailand, Ivory Coast, Haiti, and Louisiana (He et al., 1997, 2000). The fourth was from a strain of Gelasinospora, a genus closely related to Neurospora (Yuewang et al., 1996). Introngression and subsequent heterokaryosis have been used to transfer pKALILO in both directions between N. crassa and N. tetrasperma (Bok et al., 1999). Senescence plasmids may have adaptive significance for their hosts by conferring a growth advantage at high temperatures (Bok and Griffiths, 2000).

Nuclear genes also affect senescence. A recessive-lethal chromosomal gene that confers senescence was recovered from one nuclear component of a phenotypically normal heterokaryotic wild-collected N. intermedia culture (Pandit et al., 1994). This gene, sen in linkage group V, resembles in its behavior the previously described N. crassa linkage group I mutant natural death (Navraj et al., 2000). In another study, Griffiths et al. (1991) have shown that senescence caused by a mitochondrial plasmid can be suppressed by existing variants of nuclear genes.

Vegetative (heterokaryon) incompatibility. Knowledge of vegetative incompatibility has been advanced using partial-diploid progeny of insertional and terminal rearrangements to identify genes at individual vegetative incompatibility (het) loci, to study the het genes one at a time, to investigate polymorphisms in natural populations, and to distinguish multiple alleles. Additional rearrangements that are capable of detecting het loci in new segments of the genome have been described. (Perkins, 1997). Strains from nature are included in a series of reference strains used together with heterozygous partial-diploid testers to identify allelic differences at the established het loci (Perkins et al., 1993). het-c and het-6 have been cloned and sequenced (Saupe et al., 1996; Smith et al., 2000), as has the tol suppressor of mating-type-mediated vegetative incompatibility (Shiu et al., 1999). Wild-collected strains have been examined for molecular poly-

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morphism at het-c and het-6 (Powell et al., 2001; Mir-Rashed et al., 2000).

Comparison of wild strains has revealed functionally different multiple alleles at het-c (Saupe and Glass, 1997). Three het-c alleles are polymorphic and about equally frequent in a wild population of N. crassa. Other Neurospora species and fungi of related genera share these same het-c alleles, indicating that the polymorphisms were present in a species ancestral to these members of the Sordariaceae (Wu et al., 1998). The het-6 region appears to include two adjoining het loci (Smith et al., 2000). Either multiple alleles or closely linked het genes are present at another locus, het-8 (Howlett et al., 1993).

**Mating type.** Sequencing revealed that the mating type locus in N. crassa is occupied by alternative nonhomologous genes (either A or a). Because of their nonhomology, these are now termed idiomorphs rather than alleles (Glass et al., 1988, 1990). Following this discovery, mating type regions were examined and compared in different strains and species of Neurospora and in other fungi (reviewed by Glass and Nelson, 1994; Metzenberg and Randall, 1995; Staben, 1996; Coppin et al., 1997). Regions flanking the mating type locus in different Neurospora species are highly homologous except for one centromere-proximal segment that contains species-specific and/or mating-type-specific sequences (Randall and Metzenberg, 1995, 1998). DNA sequences of open reading frames from the mating type A-1 gene are highly conserved from species to species, although variation is sufficient to allow construction of an evolutionary tree for the six Neurospora species examined.

**Control of recombination.** Crossing-over in a long multiply marked chromosome segment was observed to be markedly reduced in outcrossovers between an inbred laboratory strain and unrelated wild-collected N. crassa strains. Recurrent backcrosses were used to transfer the intact marked segment into a wild strain, and crosses between parents of similar and dissimilar genetic backgrounds were then used to show that reduced recombination in outcrossovers was due to unlinked modifiers of recombination rather than to heterologies within the marked segment (Perkins and Bojko, 1992).

**RFLP mapping.** Mapping of cloned DNA segments by use of restriction fragment length polymorphisms was initiated in N. crassa by Metzenberg et al. (1984, 1985), who made available a kit of progeny obtained by crossing a laboratory strain that carried classical markers with a highly polymorphic strain from nature. The kit has been used to map over 450 RFLP and RAPD loci (see Nelson and Perkins, 2000).

**Spore killer meiotic drive elements.** Genes conferring resistance to being killed by Sk-2 or by Sk-3 were identified and shown to be linked to the Spore killer complex, which occupies a 30-unit-long interval in linkage group III (Campbell and Turner, 1987). A strain of N. crassa from the Malay Peninsula was shown to carry two additional Sk-linked genes that act together to confer resistance to Sk-2 (B. C. Turner, unpublished).

A few phenotypically abnormal progeny can be obtained from Sk-2 × Sk-3 in N. crassa. Analysis suggests that they are aneuploids originating by recombination events that also generate inviable products (Turner et al., 1988; B. C. Turner, unpublished). Sk-2 and Sk-3 were transferred into N. tetrasperma by recurrent backcrosses. Their behavior in N. tetrasperma resembles that in N. crassa (Raju and Perkins, 1991). Monokaryotic sensitive ascospores are killed, but genotypically sensitive nuclei survive in heterokaryotic (killer + sensitive) and (Sk-2 + Sk-3) ascospores. This result means that N. tetrasperma can shelter Spore killer-sensitive nuclei in heterokaryotic ascospores and that it should be capable of carrying cryptic centromere-linked Spore killer elements without any apparent effect on ascospore viability in the four-spored asci. Exceptional N. tetrasperma asci that contain recombinants between Sk-2 and Sk-3 show promise for rescuing otherwise inviable crossover products and resolving component elements of the Sk complex (Raju and Perkins, 1991).

Raju (1994, 1996) has presented criteria for distinguishing Spore killer elements from other known causes of ascospore abortion and has reviewed recent studies of meiotic drive in Neurospora and other fungi. Theoretical aspects of Spore killer drive factors in populations were considered by Lyttle (1991) and by Nauta and Hockstra (1993). Turner and Perkins (1993) list reference strains for identifying and scoring the known Spore killer elements.

Spore killer strains have been put to practical use for genetic analysis in the laboratory. Because the killer complex spans a centromere and therefore segregates from nonkiller at the first meiotic division, presence of a killer element in one parent enables physically unordered groups of eight ascospores that are shot together from the perithecium to be treated as though they were linear (ordered) asci. This method was used to obtain critical information during analysis of a segmental transposition (Perkins et al., 1995).

**Transposable elements.** Neurospora strains of differing wild origins have been important in the discovery and analysis of both active and relic transposable elements: Tad (Kinsey and Helber, 1989; Cambraeri et al., 1994), Pogo (Schechterman, 1987), Guest (Yeadon and Catcheside,
1995), and Punt (Margolin et al., 1998). Of these, the only nondefective element is the retrotransposon Tad, an active representative of which was discovered in a strain from Adiopodoumé, Ivory Coast. In an initial survey using hybridization in colony blots, only 3 of >400 strains from diverse sources appeared to contain sequences related to Tad (Kinsey, 1989). However, a subsequent survey using the polymerase chain reaction (PCR) revealed sequences closely related to Tad, not only in N. crassa, but also in numerous strains representing six other Neurospora species (Kinsey et al., 1994). These inactive Tad-like sequences contained numerous G:C to A:T mutations characteristic of repeat induced point mutation (RIP), suggesting that Tad is not of recent origin. The active Tad element is itself sensitive to RIP (Kinsey et al., 1994), and the strain in which it was discovered appears to be RIP proficient, raising the questions of how the active transposon originated and how it escaped being inactivated.

Pogo and Guest were both discovered while DNA segments were being sequenced for other purposes. Pogo has characteristic direct terminal repeats and a long internal open reading frame with sequence similarity to reverse transcriptases. Pogo-like sequences were present in multiple copies and at different genomic locations in each of three strains of different geographical origin (Schechtman, 1987, 1990). Guest has terminal inverted repeats flanked by a 3-bp direct repeat of target sequence, similar to the DNA element Tourist in maize. PCR was used to show that Guest is present in multiple copies in genomic DNA and that it has different chromosomal locations in laboratory strains of different origin (Yeadon and Catcheside, 1995).

The abundance of active transposable elements in some asexual filamentous fungi (Daboussi, 1996; Kempken and Kück, 1998) and the rarity or absence of active elements in Neurospora suggest that RIP, which operates during the sexual phase, and quelling, which silences duplicate elements during the vegetative phase, are advantageous because they prevent the proliferation of invading transposable elements (Selker, 1997).

**What Are the Prospects for Future Studies of Wild Neurospora?**

Strains described in this paper provide an opportunity to investigate some of the questions that have arisen during this study, but certainly not all. The present collection has greatly advanced what we know about Neurospora populations in nature and about species relationships. It falls far short of what is needed, however. More extensive and more systematic field studies and population sampling will be required to dispel our ignorance about many aspects of Neurospora biology. Questions such as the following remain to be answered:

- Is the life cycle completed in natural habitats in the absence of fire?
- Does chemical activation of ascospores play a significant role in nature?
- What is the role of ascospores in dispersal and colonization?
- To what extent are present-day populations indigenous? Has the genetic makeup of geographically isolated populations been altered significantly by recent introductions resulting from human agriculture and commerce?
- Do hybridization and interspecific gene flow occur when strains of different species are present on the same substrate?
- What is the extent and adaptive significance of genetically polymorphic vegetative incompatibility genes?
- Are natural populations polymorphic for genes that control circadian rhythms? Do features of the biological clock vary systematically with latitude? (Media have only recently been devised that enable free-running period lengths to be determined for unaltered isolates from nature [Morgan and Feldman, 1998].)
- What is the past history and present role of the many defective transposable elements that are present in the Neurospora genome, especially in centromere regions? Are active transposons introduced from other species, only to be inactivated by RIP?

Other problems that bear on evolution also remain to be solved:

- Are cryptic species present within what we have defined as species on the basis of mating behavior? That is, does a single biological species of Neurospora include subpopulations that would meet the criteria for definition as distinct phylogenetic species if evolutionary trees are constructed using molecular homologies?
- What are the consequences of pseudohomothallism for the population genetics and evolutionary biology of N. tetrasperma?
- What is the significance of blocked recombination in the N. tetrasperma mating type chromosome, and how did it originate?
- Why does the sexual cycle persist in the homothallic Neurospora species, despite the frequent occurrence of
mutations that impair fertility? Are ascospore dormancy, the role of ascospores in dispersal, and the ability of meiotic recombination to prevent accumulation of multiple recessive-lethal mutations significant factors?

- Are asexual derivatives of Neurospora present in nature, and could they provide a safe haven for RLP-vulnerable transposable elements such as Tad? Might imperfect forms of Neurospora that lack conidia and ascospores be identified by a survey of soil samples using the polymerase chain reaction?
- What is the incidence and what are the evolutionary consequences of genetic elements that show meiotic drive? Are drive elements present that have become fixed in some local populations but not in others or that are difficult to detect because they differ from the known Spore killer complexes in showing a lower level of segregation-ratio distortion?

Both local and global studies are needed. Selected local populations should be used over extended periods for observations and experiments addressing such questions as the stability of genetic polymorphisms and the dynamics of introduced drive elements, retrotransposons, and senescence plasmids. The global study would extend sampling of natural populations beyond what we have been able to do. New geographical areas should be explored, especially oceanic islands. Most parts of Africa, South America, and Indonesia are still unrepresented. Extended sampling should provide information on geographical distribution and ecology and augment the repertory of Spore killers, heterocarps, mating type flankers, and genetic variants of other types. New species might also be discovered.

Findings with the wild strains and the ways that they have proved useful in the laboratory have far exceeded what we anticipated when the study was initiated in 1968. However, much remains to be done, and even with the many strains already in hand, there is little indication of diminishing returns or significant redundancy as additional populations are sampled and additional cultures are acquired.

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