Fungal biology in the 21st century

Ramesh Maheshwari

Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

Fungi are a diverse group of organisms comprising both single-celled and multicellular filamentous forms. It has been estimated that only a fraction of the diversity that exists is presently known. In the 20th century several species, each with its own special advantages, were introduced in research as simplest eukaryotic model systems that can be studied with the approaches of cell biology, genetics and biochemistry. The genome sequences of a few fungi are now known; those of several other species are underway. In the 21st century, fungi will not only be increasingly used for understanding their unique mode of life, but also for findings of general applicability to higher organisms, such as assembly of intracellular organelles, adaptation to harsh environmental conditions, defence mechanisms for protection from invasion by foreign DNA, biological rhythms, aging and death. Their ability to be transformed and the transgenic strains to be grown in relatively simple nutrient medium in industrial-sized fermentors, and their extracellular secretion of proteins is likely to be exploited for production of a variety of enzymes (proteins), including human vaccine.

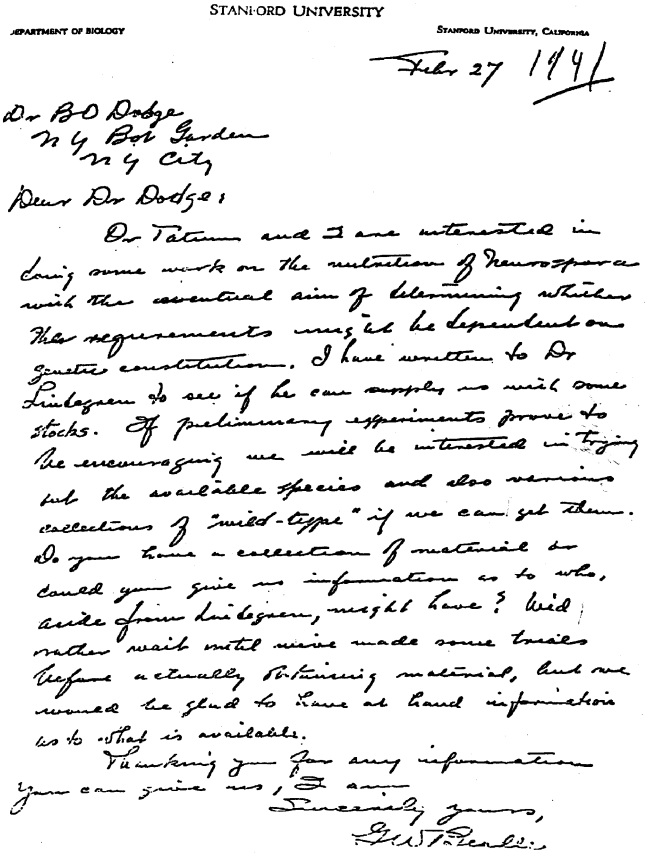
The fungi are progressive, ever changing and evolving rapidly in their own way, so that they are capable of becoming adapted to every condition of life. We may rest assured that as green plants and animals disappear one by one from the face of the globe, some of the fungi will always be present to dispose of the last remains.

—B. O. Dodge (1872–1960)

FUNGI are non-photosynthetic, eukaryotic organisms which grow as single cells (yeasts) or as multicellular filaments (moulds/fungi), acquiring nutrition by absorption from their surroundings. There is no material of biological origin that remains free of fungi. Although commonly and unpleasantly thought of as causing spoilage of stored food and diseases in plants, the large majority of fungi decompose dead material and recycle essential mineral nutrients (particularly nitrogen, phosphorus and potassium) required to build the cytoplasm. Fungi thus contribute to the green cover on earth[1]. Some fungi live in plants as endophytes (symptomless parasites)[2], or as symbiotic partners with algae (lichens), enabling them to grow under harsh conditions which they could not do otherwise. A few fungi are opportunistic human pathogens[3]. Since antiquity, yeast has been exploited unwittingly for the conversion of grape juice into ethanol and for making bread. Some fungi are sources of life-saving drugs, such as penicillin and cephalosporin. Species of fungi are the only eukaryotic organisms that thrive at a temperature range (45–60°C)[4] at which no plant or animal can live. Although their individual hyphae are hard to see by unaided eye, fungi are the largest living organisms[5], rivalling the mass of a blue-whale, a California redwood tree or a jumbo jet[6]. Here, I give an overview of current trends in biology of fungi and a wish-list of some future research problems.

Spotlight on fungi

Simple growth tests by Beadle and Tatum with a *consciously* selected fungus (Figure 1) that grows rapidly on a simple, chemically-defined medium, and whose haploid cells (conidia) can be irradiated to generate auxotrophic mu-



STANFORD UNIVERSITY

DEPARTMENT OF BIOLOGY — STANFORD UNIVERSITY, CALIFORNIA

Feb 27 1941

Dr B O Dodge
N Y Bot Garden
N Y City

Dear Dr Dodge:

Dr Tatum and I are interested in doing some work on the nutrition of Neurospora with the eventual aim of determining whether the requirements may not be dependent on genetic constitution. I have written to Dr Lindegren to see if he can supply us with some stocks. If preliminary experiments prove to be encouraging we will be interested in trying out the available species and also securing collections of "wild-type" if we can get them. Do you have a collection of material so could you give us information as to who, aside from Lindegren, might have? Wild rather wait until mine made some tests before actually obtaining material, but we would be glad to have at hand information as to what is available.

Thanking you for any information you can give us, I am

Sincerely yours,

G W Beadle

Figure 1. The choice of any species of fungus in biology results from prior studies of that fungus – A letter from G. W. Beadle to B. O. Dodge requesting a culture of *Neurospora crassa.* Reproduced by permission from New York Botanical Gardens.

e-mail: fungi@biochem.iisc.ernet.in

tants (strains that require nutritional supplements), led to the discovery of the relationship between genes, protein and phenotypes – known as the 'one gene–one enzyme' hypothesis[7]. The use of temperature-conditional mutants of yeast by Hartwell led to the identification of genes that control the fundamental process of cell division[8]. These discoveries, recognized by Nobel Prizes, brought together genetics and biochemistry. Geneticists and biochemists are being joined by physicists to determine the three-dimensional structures of proteins and their interactions in order to understand the molecular design of life. If a choice exists between solving the same problem, it makes sense to choose an organism which can be grown rapidly, economically and can be manipulated by the techniques of genetics and molecular biology. Fungi, such as yeast or *Neurospora*, have become established as the simplest eukaryotic models for findings applicable to organisms of greater complexity[9].

Teaching and research

Because fungi possess cell wall in common with plants, the Swedish botanist Carolus Linnaeus (1707–78) included fungi in plants. Fungi are therefore generally taught in botany. Note that Beadle and Tatum published their epochal research in a botany journal! But, fungi have more in common with animals than with plants: (i) both lack chlorophyll, (ii) both commonly have exoskeleton (wall) containing chitin, (iii) the typical sugar in both is trehalose (an α, 1–1 diglucoside) which is absent in plants, (iv) the polysaccharide reserve in both is glycogen and not starch that is found in green plants, (v) the amino acid sequences of some proteins (the elongation factor 1a, actin, alpha and beta tubulins, enolase) and the nucleotide sequences of ribosomal RNA are closer to animals than to plants[10,11]. Although their single spore or hypha is of microscopic dimension, microbiologists are skeptical about placement of fungi, essentially because of their eukaryotic nature (DNA packaged into distinct chromosomes; a cell cycle similar to plant and animal cells; presence of membrane-bound organelles; a multilayered cell wall; larger size (80S) of their ribosome, etc). Based on a comparison of the mode of nutrient acquisition (injestion, absorption or photosynthesis), Whittaker[12] proposed a five-kingdom classification of the diversity of life and gave fungi a kingdom of their own. Even though his scheme of classification has been widely accepted, and fungi outnumber all organisms excepting insects, mycologists (those who study fungi) have not demanded separate departments.

Trends in fungal biology

Taxonomy

Ever since fungi began to be studied nearly two centuries ago, new species have been described, although the discovery rate has steadily declined. Is this because today fewer scientists are engaged in exploration, collection, identification, naming and classifying fungi, or is it because the majority of fungi have already been discovered? Hawksworth[13] estimated the number of fungi that occur globally, based on the ratio between the known species of plants and fungi in well-studied regions. This ratio is 1 : 6 for the UK, 1 : 4 for Finland, 1 : 4 for Switzerland, 1 : 1 for USA, and 1 : 0.5 for India – the latter is undoubtedly due to under-exploration of the diverse environments of the subcontinent. Applying the 1 : 6 factor to the global total of 250,000 species of plants, the total number of fungi approximates to 1.5 million, making fungi the second-most abundant group of organisms, next only to insects. However, only 5% of this number is actually documented. Where are the undiscovered species? Anywhere, where moisture and nutrients for the synthesis of protoplasm are available, but more likely in the tropics where there is a greater diversity of flora and fauna and micro-habitats. Fungi occur in the most unexpected places (Table 1). Hitherto regarded as strictly aerobic, fungi have been found even in the rumen of herbivores[14], assisting in the digestion of cellulose.

Biologists of the reductionistic bias ask: why is the study of fungal diversity important? David Perkins answered this frequently asked question thus[15]: 'Knowledge of a flute or a kettledrum is not sufficient to understand all the other instruments in a symphony orchestra or to predict their characteristics. Nor is knowledge of a single species, however complete, adequate for understanding diverse species. Diversity of research organisms in the laboratory must at least dimly reflect the diversity of species in nature, if the scope and the beauty of evolutionary improvisations are to be appreciated and the genetic manipulation that brought them about are to be understood'. A fungal species may be a source of a new drug, a new antibiotic, or an enzyme-variant resistant to harsh conditions of pH, temperature or end-product inhibition. Recall that the demand for a substitute for rennin, obtained from stomach of calves, led Japanese scientists to isolate and screen several hundred microorganisms and select a fungus which produced a thermostable acid protease for curdling milk in the manufacture of cheese[16]. There is an undiminished demand for alkaline lipases and proteases in the manufacture of enzyme-fortified detergents for removing oil and sweat stains from garments in hot-water machine wash. There is a demand for thermostable amylases for conversion of starch into glucose at high temperature to reduce the risk of contamination. Unusual fungi from unusual places have been found to produce taxol – a drug with anticancer properties. Scientists had proposed the manufacture of ethanol in a two-step process by hydrolysis of cellulose into glucose using cellulase enzymes produced by moulds, and converting glucose into ethanol by fermentation using yeast. Constant hikes in fuel prices entail a serious reconsideration of the use of gasohol (a mixture of petrol and

Table 1. Examples illustrating diversity of fungi and their habitats

Term used	Meaning of the term	Example(s)
Anthropophilic fungi	Infectious only to man	*Trichophyton rubrum*
Aquatic fungi	Fungi resident in aquatic habitats	*Saprolegnia*
Bryophilous fungi	Fungi occurring on bryophytes	*Thyronectria hyperantartica*
Coprophilous fungi	Fungi growing on dung of herbivore animals	*Pilobolus, Podospora, Coprinus*
Corticolous fungi	Fungi growing on tree bark	*Xylaria* sp.
Dermatophyte	Fungi that live as parasites on skin, hair, or nails of man and other animals	*Trichophyton interdigitale, Microsporum canis, Arthobotrys* sp.
Endolithic fungi	Fungi living inside rocks or stony material	*Pyrenocollema halodytes* (syn. *Pharcidia balani*)
Endophytic fungi	Symptompless parasitic fungi in mutualistic association with living plants	*Balansia* sp., *Curvularia* sp.
Entomogenous fungi	Insect-parasitizing fungi	*Entomophthora, Cordyceps, Septobasidium, Beauveria*
Halotolerant fungi	Fungi tolerating 7 to 15% salt	*Aspergillus* sp., *Gymnascella marismortui Tuber* sp.
Hypogeous fungi	Fungi growing below ground	
Keratinophilic fungi	Fungi growing on feathers, horns	*Onygena equine, Nannizia*
Lichen forming fungi	Fungal symbiont of lichen thallus	*Peltigera* sp., *Cladonia cristellata, Xanthoria* sp.,
Marine (saprobic) fungi	Fungi growing and sporulating in marine or estuarine habitats	*Dendryphiella salina, Mycosphaerella*
Mesophilic fungi	Fungi thriving between 10 and 40°C	Vast majority of fungi, e.g. *Aspergillus niger*
Mycorrhizal fungi	Fungi in symbiotic association with living roots	Mostly basidiomycetous fungi belonging to families Agaricaceae, Boletaceae
Mycoparasites	Fungi parasitic on other living fungi	*Trichoderma* spp., *Piptocephalius* sp., *Gliocladium roseum*
Nematophagous	Fungi parasitic on nematodes	*Arthobotrys* sp., *Dactylaria* sp.
Osmotolerant fungi	Fungi capable of growth in solutions of high osmotic pressure	*Aspergillus restrictus, A. flavus, A. amstelodami*
Psychrophilic fungi	Fungi growing at <10°C, maximum temperature of growth at 15–20°C	*Mucor* sp., *Fusarium nivale, Typhula idahoensis*
Pyroxyrophilous fungi	Fungi growing on burnt trees, wood or burnt areas of the ground	*Anthracobia* sp., *Pyronema* sp., *Daldinia* sp.
Resinicolous fungi	Fungi colonizing resin exuded from coniferous trees	*Chaenothecopsis* sp, *Claussenomyces* sp, *Myocalicium* sp.
Rumen fungi	Fungi in anaerobic environment of rumen of herbivores	*Neocallimastix frontalis*
Sewage fungi	Fungi growing in polluted waters	*Leptomitus lacteus, Fusarium aqueductuum*
Thermophilic fungi	Fungi which can grow at 45°C or above, but not at 20°C	*Thermomyces lanuginosus, Mucor miehei*
Water moulds	Fungi found in waters	*Saprolegnia, Achlya, Dictyuchus*
Xerotolerant fungi	Fungi growing on jams, salty foods at <0.85 aw	*Aspergillus fumigatus, Cladosporium* sp.

ethanol) and a search for powerful sources of fungi capable of breaking down lignocellulosic material – nature's only renewable resource. Here I recall the words of the Indian mycologist, Subramanian[17], noted for his discovery of several new species of fungi: 'All fungal biotechnology begins with a preferred fungal genome drawn from nature – a natural species and its numerous genetic strains. The genomes for manipulation must come from the mycodiversity extant in the biosphere. The enumeration, identification, isolation, maintenance in culture, and conservation of these genomes in the biosphere is therefore the most urgent and vital single task for mycologists, biotechnologists, biologists, naturalists, environmentalists or whatever one may like to call oneself – but essentially for Man and for Science'. A concern is that the expertise in collecting samples, isolating, culturing and identifying fungi is almost lost in the molecular era.

Redefining and classifying fungi

Fungi have traditionally been classified based on microscopic features such as the size, shape, surface ornamentation of spores, their mode of formation, etc. Nowadays, classification is increasingly based on quantitative estimates of homology of sequences of ribosomal RNA or DNA. Recall that in 1845, the potato leaf blight fungus *Phytophthora infestans*, had totally destroyed the potato crop, once the staple diet of the Irish people, resulting in almost a million deaths and the mass migration of people to USA and other countries. Soon after, another fungus *Plasmopara viticola* had threatened the wine industry in France, but luckily the pathogen was controlled by the chance discovery of Bordeaux mixture (a mixture of copper sulphate and lime) that was sprayed on plants to discourage pilferage of grapes. These two fungi, and their close allies, which had catalysed the science of mycology and plant pathology, are now considered not to be fungi because of their different sequences of 16s rRNA molecule found in the small subunit of ribosome of all organisms. Some mycologists call these as 'pseudofungi' and place them into a new domain of life called Straminipila[18,19]. This has created much confusion and has been criticized by traditional mycologists as 'molecular myopia'. The counter-argument is that Straminipila produce flagellated (motile) spores called zoospores and

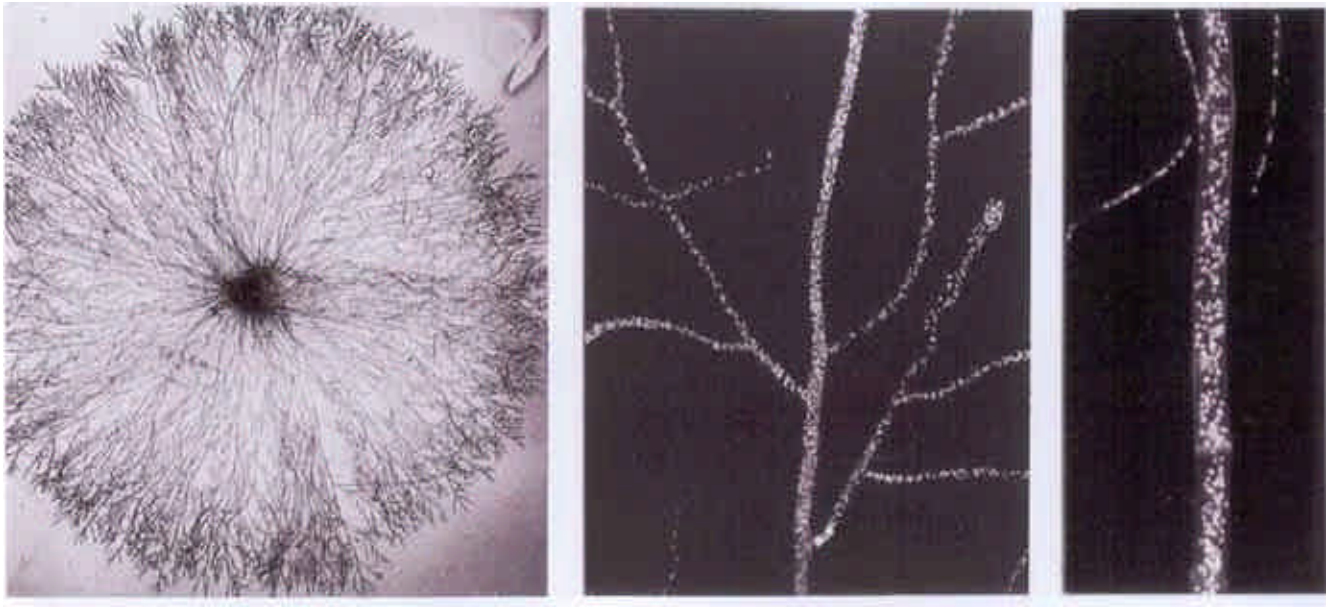


Figure 2. *Neurospora crassa*. (Left) One-day-old colony growing on agar medium. (Centre) Portion of mycelium stained with a DNA-binding fluorescent dye to visualize nuclei, and with a chitin-binding fluorescent dye to visualize septa. (Right) Enlarged view.

are, therefore, derived from an alga that had lost its chloroplasts. Straminipila also differ from the majority of moulds in being diploid (each chromosome present in duplicate) rather than haploid (each chromosome present singly), and in containing cellulose rather than chitin as a major component of the cell wall. This situation is reminiscent of certain forms of life, hitherto identified as bacteria, being separated into the domain Archaebacteria[20], renamed Archaea. I suppose that a debate on 'What is a fungus?' will revive interest in comparative morphology, cytology and cellular chemistry, and lead to new ideas on classification of microorganisms and evolution of fungi.

Mechanisms of polarized growth

How does the fungal hypha develop in the form of a microscopic tube of even diameter (Figure 2), and how are the sites and the time of lateral branches selected? This question is basically asking how plants or animals establish an *axis* – a root end and a shoot end or a head and a foot. A unicellular fungus, *Saccharomyces cerevisiae* (budding/brewer's yeast), is providing clues on the core mechanisms involved[21,22]. At every division cycle, the yeast selects the site of a new bud in a spatially distinct pattern. Haploid cells choose bud sites in an axial pattern in which mother and daughter cells bud adjacent to their prior mother-bud junction, while the diploid cells bud in a bipolar pattern with the buds arising either adjacent to the last daughter cell or at the pole opposite the last daughter cell. The two distinct patterns of budding are manifestations of cell polarity, defined as asymmetry in cell shape. The critical steps in establishment of cellular polarity are easily identified by microscopy of temperature-sensitive mutants in which the growth is reversibly arrested by a temperature change. The mutational approach has revealed that the machinery involves a number of proteins for critical delivery of membrane and cell-wall precursors for polarized growth. Bud growth is initiated by marking the potential bud site on the mother cell by re-orienting the cytoskeleton (actin cables) at the site to guide the delivery of Golgi-derived vesicles containing membrane and cell-wall precursors for localized docking and fusion to the membrane growth site. However, critical questions remain unanswered: how is the point in the cell for bud growth selected and how is the cytoskeleton oriented for delivery of vesicles to that site?

Phase contrast microscopy has shown that the growing fungal hypha has a unique apical body called Spitzenkörper (in German). Spitzenkörper is observed at the tip of the growing hypha, below the plasma membrane. It is also seen at the tip of lateral branches prior to their fusion, suggesting that this structure delivers digestive enzymes for formation of a fusion pore at the point of contact of hyphal tips and of cell-wall precursors for interconnecting hyphae[23,24]. Does Spitzenkörper also determine the direction of growth of hypha[25]?

Multinuclear condition and heterokaryosis

In fungi, nuclear division and cytokinesis are not obligatorily coupled. Consequently, even if formed from a single uninucleate spore, the hypha becomes multinuclear, raising the question as to what advantage accrues to fungi from multinuclear condition, whereas cells in complex forms (plants and animals) have just one nucleus per cell?

Paradoxically, although nuclei are bathed by a common cytoplasm, their divisions are not synchronous[26,27], suggesting that fungal nuclei control their division independently. A recent study has even questioned if all nuclei in a fungal hypha are simultaneously active and contribute to the phenotype[27]. This may be testable by a technique which can measure the transcriptional activity of individual nuclei *in situ*.

A consequence of the multinuclear condition is heterokaryosis (i.e. the existence of two or more genetically different nuclei in the same cell). Although mutation rate is estimated to be in the order of one in million nuclei, the likelihood of a mycelium that contains thousands of nuclei becoming heterokaryotic due to accumulation of spontaneous mutations must be rather high. Our knowledge of biology was gained almost entirely from the study of organisms having one nucleus/cell. Will the fungi spring surprises? For example, when a heterokaryotic fungal cell is transformed, the transforming DNA enters into only one type of nucleus at a time, rarely into both nuclear types[28,29], indicating that the nuclear types are not simultaneously 'competent' for the uptake of introduced DNA. Puzzling cases of severe competition or conflict between the nuclei have been discovered[30], similar to that in populations of animals or humans. The mycelium can be thought of as a population of nuclei in which the properties of variation, drift, migration, mutation, competition and selection prevail[9].

Dynamics of organelles and molecules

A recent advance has been in visualizing structures in living cells that had previously been seen only by microscopy of killed cells. The hyphae which can be miles long[5,31], are excellent material for studying the long-distance movement of organelles and molecules. Nuclei, tagged with green fluorescent protein (GFP) have been used to monitor changes in shape and their movement by video-enhanced fluorescence microscopy[32]. Nuclei move in opposite directions in the hyphal compartment to reach a branch initial, suggesting individual regulation of nuclear movement. Velocities from 0.1 to 40 $\mu m\ min^{-1}$ have been observed. Fungal mutants have provided evidence for a track for the nucleus to move, a molecular motor to pull it, and a coupling mechanism to link the motor to nucleus[33–35]. Identification of motor molecules that move nuclei and other membrane-bound organelles at different velocities and at different positions is becoming a hot topic and has a parallel in animals too – synaptic vesicles are transported in the long extensions (axons) of the nerve cell for normal functioning of nerves. The analysis of structures of specific motor proteins that move different cargoes, and of the mechanisms involved is an exciting area of research. Freitag *et al.*[36] used *b*-tubulin-GFP to visualize polymerization and depolymerization of microtubules, and histone-GFP tagged nuclei to study diffusion of protein molecules and silencing of nuclei in a common cytoplasm.

Is novel gene regulation possible by adjustment of inter-nuclear distance? Microscopy of hyphae shows nuclei as well-spaced or clustered. Schuurs *et al.* [37] have suggested that spacing of nuclei–whether juxtaposed or separated–may signify a unique gene regulatory mechanism in fungi. In a mushroom fungus, *Schizophyllum commune*, the type of hydrophobin (proteins rich in non-polar amino acids which give fungal fruiting body and spores their water-repelling property) could be modulated by internuclear distance.

Developmental genetics

Many fungi produce mitotically-derived asexual spores on a conidiophore – a morphological device for the rapid production of a large number of conidia in a small space for effective dissemination by air current or splash of rain, or insects. *Aspergillus nidulans* illustrates the basic strategy of asexual reproduction. The conidiophore of *A. nidulans* is a multicellular structure in which the cells (metulae and phialides) are symmetrically arranged, producing a chain of conidia vertically with great economy of space. The cell types develop in an orderly manner, in precisely timed sequence: undifferentiated hyphae (0 h) $\rightarrow$ aerial stalk (5 h) $\rightarrow$ vesicle (10 h) $\rightarrow$ metula and phialide (15 h) $\rightarrow$ immature conidia (20 h) $\rightarrow$ mature dark green conidia (25 h). Through characterization of mutants that show severe phenotypic alteration, three genes have been proposed to define a central regulatory pathway *brlA* $\rightarrow$ *abaA* $\rightarrow$ *wetA* that controls the expression of conidiation-specific genes. The temporal sequence of steps suggests that master regulatory genes are involved[38]. The complete genome sequence of *A. nidulans* will make it possible to determine the number of genes from the open reading frames. It is expected that genes with a role in sporulation will be analysed using DNA microarrays to determine whether the physical linear order of the genes is related to the time of their expression, the number of clusters of co-expressed genes, and gene expression in known mutants that show severe phenotypic alterations.

A contentious question is whether reproduction is induced by nutrient starvation or is it an expression of an inbuilt development programme, only indirectly influenced by nutrient availability[39]. Forced expression of conidiation genes, using an inducible promoter fused to a regulatory conidiation gene, in a fungus grown in non-limiting nutrient condition will allow this to be assessed.

Biogenesis of mitochondria

Rather than soft cells without walls (such as from beef heart or horse muscle), how is it that fungi with their tough cell walls to disrupt are the choice material for investigations on biogenesis of an intracellular organelle? It had been claimed that mitochondria appear and disappear in

yeast when it is grown in the presence or absence of oxygen. Schatz[40] showed that mitochondria in yeast are *permanent* structures having a constant amount of DNA, although the amount is not enough to code for the many proteins in the mitochondrion. This puzzle encouraged development of methods for isolating mitochondria from fungi, and to determine how mitochondrial and nuclear DNA cooperate in the control of mitochondria formation[41]. The majority of mitochondrial proteins are specified by nuclear genes and synthesized in the cytoplasm from where they are imported into the organelle. In the [*petite*] mutant of yeast and the [*poky*] mutant of *Neurospora*, growth abnormalities are inherited maternally (cytoplasmic inheritance), implicating that nuclear–mitochondrial interactions are modified resulting in abnormalities. Both nuclear and mitochondrial genes function together in assembly mitochondria[42]. In *Neurospora crassa*, a novel genetic technique (sheltered RIP in essential genes) allows the maintenance of mutated alleles in a heterokaryon in which the normal copy of the gene, present in another nucleus, shelters the cell against potentially lethal effects of mutations[43]. This allows the role of individual proteins of the multi-protein translocase machinery in the outer membrane and in the inner membrane to be evaluated for sorting proteins destined for the outer membrane, the inner membrane, or the matrix.

Fungal senescence – a paradigm for mitochondrial diseases in humans

Fungi are potentially immortal[5,31]. However, some wild strains of *Podospora anserina*, of *N. crassa* and *N. intermedia* progressively lose vigour and die upon subculturing, regardless of the composition of the medium – a phenomenon termed senescence[44]. Fungi are attractive material for investigation of senescence since the senescing strains can be rendered 'permanent' by lyophilization, or by cryopreservation and revived for experimentation when desired without losing the entire stock of culture. Alternatively, a senescence strain may be preserved indefinitely by fusing it with a normal (wild-type) strain in the form of a heterokaryon from which the senescing nuclear type is recovered by conidial plating, avoiding permanent loss of the genotype. Reciprocal crosses have shown that determinant of senescence is either in the nucleus or in the cytoplasm (mitochondria). Senescence in the single-gene nuclear mutants, *natural death* (*nd*)[45,46] and *senescent* (*sen*)[47,48] of *N. crassa* is associated with large deletions and sequence rearrangements of mitochondrial DNA due to a high frequency of mispairing and crossing over between homologous sequence repeats resulting in respiratory defects, suggesting that protein products of wild type nd^+ and sen^+ genes protect the mitochondrial genome from deletions and illegitimate recombination events that apparently occur by default because of palindrome sequence repeats. Cloning nd^+ and sen^+ and identification of gene products is important not only in understanding the assembly of mitochondria, and the maintenance of mitochondrial genome by nuclear-encoded protein factors, but also for identifying human homologues of mitochondrial diseases[49]. Populations of *Neurospora* have senescence-inducing mitochondrial plasmids which disrupt mitochondrial energy production by insertional mutagenesis[50,51]. Apart from focusing attention on the role of extrachromosomal genetic elements in the etiology of diseases, the high similarity of plasmid DNA sequences raises questions on their origin and the mechanism by which they have become globally distributed in natural populations.

Cell–cell recognition and sexual development

Fungi too indulge in sex. However, in fungi the mating partners may not be morphologically differentiated. Conjugation may occur between cells containing genetically identical (sister) nuclei on neighbouring hyphal branches of the same individual. Fertilization and meiosis are still involved, raising the question as to why sexual reproduction persists when they can also reproduce by mitotically-produced cells (conidia). There are many fungi in which only asexual reproduction is known; but there are also many fungi which reproduce only sexually. Fundamental questions arise: How, among a large number of individuals in their surroundings (soil), do the potential mates find partners, and coordinate their choices in an accurate way and conjugate? The corn smut fungus *Ustilago maydis* exemplifies several features of sexual development in fungi. The recognition of haploid cells (conjugants) is based on pheromones which are small size polypeptides with a farnesyl group attached, that orients the growth of cells for contact and for 'commitment'. There are hundreds of different genetically determined mating types (individuals). The mating types regulate the choice of mates despite lack of morphological differentiation. The *a* locus regulates cell fusion and has two alleles, each allele contains two genes, one for a pheromone polypeptide and one for a pheromone receptor[52,53]. It is therefore the determinant of cell–cell recognition. The *b* locus controls nuclear fusion. The necessary condition for a successful mating is that two nuclei must have two different alleles. The *b* locus has a pair of divergently transcribed genes, *bE* and *bW*, whose nucleotide sequences suggest that they encode homeodomain proteins that bind to DNA and function as transcription factors. A yeast two-hybrid system was used to demonstrate that one *bE* and one *bW* can associate into a dimer, but only if they are derived from different alleles. To what DNA sequences does the transcription factor bind, resulting in nuclear fusion and meiosis, remains to be determined.

Host defence mechanisms (gene silencing)

The majority of fungi are saprophytes, existing among dead organisms. They are therefore vulnerable to assault

by homologous or heterologous DNA leaking out from dead cells in their environment. Fungi have evolved surveillance and protection mechanisms for maintaining their genomic integrity. Transformation procedures have been standardized for several fungi to study the fate of engineered DNA molecules introduced inside the cell. The fungus *Neurospora* is a favourite organism for these studies because of its bright-orange colour (Figure 3) and well-developed genetics. Will the colour of the fungus be intensified by the introduction of extra copies of carotenoid genes, or will the expression of both the resident and the introduced genes be silenced? The ability of vegetative cells to fuse to form a heterokaryon allows investigation of the interactions between silenced and non-silenced nuclei in the mycelium. A variety of gene-silencing phenomena discovered, in chronological order, are: (i) the duplicated DNA sequences are inactivated by mutation in the meiotic phase, a process known as RIP (repeat-induced point mutation)[54], (ii) the duplicated DNA sequences during meiotic phase are inactivated by methylation, a process known as MIP (methylation-induced premeiotically)[55] , (iii) multiple copies of transgenes in the vegetative phase are irreversibly inactivated and silencing is dominant in heterokaryon, a process called quelling[56] (Figure 4) , (iv) silencing is maintained even in the absence of the transgene[57], and (v) silencing of transgene which is in an unpaired state in the sexual phase occurs, by a process called MSUD (meiotic silencing of unpaired DNA)[58]. The generality as well as details of these processes require to be understood. For example, how premeiotic cells recognize the presence of extra copy of chromosome segment? How does DNA methylation repress transcription?

Molecular plant pathology

Many plants constitutively produce triterpenoid, steroid or steroidal glycosylated compounds to protect themselves from predators and pathogens, which are generally inhibitory to fungi. These molecules are known by the general term saponin because of their soap-like properties, derived from the plant *Saponaria officinalis*, the extracts of which were used to make soap[59]. Saponins make complexes with membrane sterols, resulting in pore formation and leakage of cell constituents. Saponins may provide a general defence mechanism against fungi. Not surprisingly, only 2–3% fungi are pathogenic. The leaves and green fruits of tomato contain high levels a steroidal glycoalkaloid called tomatine. The pathogenecity of *Septoria lycopersici* on tomato was attributed to the production of a glycosyl hydrolase, tomatinase which detoxifies tomatine by removing a single terminal glucose molecule by hydrolysis of a ***b***, 1–2 linkage[60]. The targetted gene-disruption technique was used to test the role of saponins in pathogenecity. The identification of saponin detoxifying enzymes and their confirmatory role in pathogenesis by gene disruption approach is expected to be another active area of research.

Rather than locating a portal for entry into the host by chance, plant pathogenic fungi have evolved a highly sensitive mechanism of 'touch and feel' that guides the germ tubes towards the stomata for entry inside the host[61,62]. The thigmotropic signal is translated into a morphogenetic programme that results in sequential differentiation of specialized cells (infection structures) and ultimately to produce a special absorbing cell called haustorium. It is through the interface between the host cell and the haustorium that molecular information is exchanged and nutrients are absorbed. The pathogen acts as a sink by modifying the normal pattern of translocation of photosynthate within the host tissue. A major goal will be the clarification of the host–parasite interface, characterization of the nutrient transporter systems in haustoria, and the dissection of the signalling pathway in a compatible interaction that results in the diversion of host resources to the fungus[63]. The new understanding that the transition from germ tube to mycelium proceeds through formation of infection struc-

Figure 3. *Neurospora* on sugarcane stubble after post-harvest burning of agricultural field in Karnataka. Because of carotenoid pigment the fungus is easily recognized in nature and has become a model for studies on population genetics and speciation.

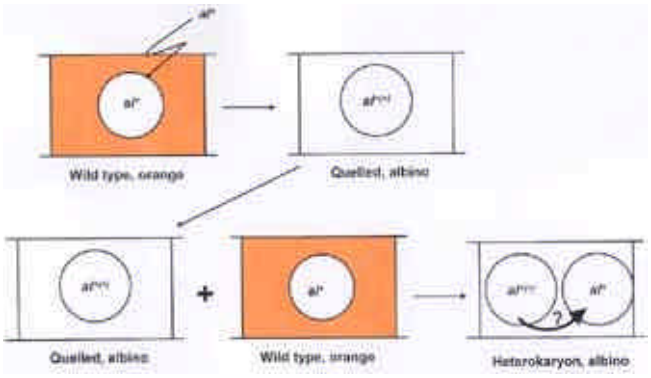


Figure 4. Gene silencing (quelling) in heterokaryon of *N. crassa*. The rectangle is a hyphal cell with only one nucleus of each genotype shown as a circle. The genotype of wild-type nucleus is indicated as *albino*$^+$ (abbreviated as *al*$^+$). The gene duplicated is shown by curved arrow. The extra copy of gene introduced by transformation is shown in parenthesis. The phenotype is shown as orange or white.

tures, formed from contact with hydrophobic, ridged surface of precise geometry, and that the biotrophic fungus can take in nutrients only through haustoria may be important in their culture on artificial media, leading to molecular studies.

Molecular biology of human pathogenic fungi

A few species of fungi cause allergy and diseases in man. *A. fumigatus*, a fast-growing saprophytic, thermotolerant and high sporulating fungus produces airborne conidia which reach the lung by inhalation and cause aspergillosis in patients receiving immunosuppressive therapies[64]. Strain typing has revealed extreme genetic diversity in this fungus. Research is being carried out to determine the putative fungal virulence factors that stimulate mycelial growth and/or survival in the lung based on the analysis of mutants. A genome sequencing project has been launched (http://www.aspergillus.man.ac.uk) for identification of molecular features that favour the mycelial growth in human tissues using experimental mouse system. A pigmentless-conidium mutant with altered conidial surface and reduced virulence will stimulate studies of factors required for adhesion. *Candida* species constitute the most common cause of nosocomial blood stream infections and of pneumonic mortality in bone marrow/stem cell transplant recipients[65]. *Histoplasma capsulatum* is the common cause of fungal respiratory infection. Some pathogenic fungi, including the human pathogens, *C. albicans*, *H. capsulatum*, *Paracoccidioides brasiliensis*, and the plant pathogen, *Ustilago maydis* are dimorphic, i.e. they switch from saprophytic yeast to pathogenic mycelial phase[66]. An intriguing question is what controls the switch from the mycelial form to yeast form? Genomic microarrays using a cell culture model of macrophage infection are now being used to identify phase-specific genes, its dual lifestyle and the genetic basis for its pathogenicity. The genes controlling morphogenesis are potential targets for novel antifungal drugs. The Whitehead Institute/MIT Center for Genome Research (WICGR) proposes to compare the genome sequences of these and other non-pathogenic fungi (e.g. *N. crassa*) to define the genetic differences in the pathogens that contribute to infection and diseases. Antifungal targets are focused on synthesis of fungal cell wall (*b*-1,3 glucan) and membrane sterol (ergosterol).

Mycorrhizal fungi

Roots of nearly 90% plants form a symbiotic association with fungi called mycorrhiza ('fungus root'). Contrary to popular belief, the luxuriance of rainforests is not because the rainforest soil is more fertile (as torrential rains over millennia leach out soluble minerals), but because the roots associate with fungi whose spreading hyphae increase the area of absorption of scarce nutrients and transport these to the plant in return for photosynthetically fixed carbon. In the symbiotic interaction, the fungus enters the root cells to form specialized haustoria called arbuscules because of their highly branched, tree-like structure. Arbuscular mycorrhizal fungi also develop an extensive hyphal network external to the plant root, which provides the physical link between soil and root, drawing phosphorus and other minerals from the soil and translocating them to the root. The mechanisms that are responsible for the increased uptake from soil and transfer to host through the interface need to be identified. A proteome analysis based on separation of proteins by two-dimensional electrophoresis and their identification by mass spectrometry has been initiated to identify proteins involved in mycorrhizal development and functioning[67].

Biochemical adaptations

Even for the seemingly most unlikely substrata, there is usually some fungus that can decompose them. Were it not for some reports by some esteemed mycologists, it would be hard to believe that a few entomogenous fungi are specific for the sex or even the position (left or the right side) of the host insect! It would be a challenge to culture these fungi, study their morphogenesis, pathogenesis, reproduction and dissemination, and the basic mechanisms and strategies in adaptation.

Because the mycelium is hidden inside the substratum, few studies have been done to understand the physiological and biochemical means of adaptation to environment. To give an example: contrary to expectation, invertase in thermophilic fungus is a highly unstable enzyme and requires a thiol compound for keeping essential sulfhydryl group(s) in protein molecule in the reduced state for catalytic activity[68,69]. The strategy evolved is to keep the enzyme in the hyphal tip which has a reducing environment. Moreover, unlike in mesophilic fungi, invertase in the thermophilic fungi is inducible – it is rapidly co-induced with sucrose transporter *only* when its substrate (sucrose) is available in the environment, thereby saving on energy if the enzyme were to be synthesized constitutively regardless of the availability of sucrose in the environment.

WICGR has released sequences of over seven filamentous fungi (www.genome.wi.mit.edu/annotation/fungi/). It is hoped that representative fungi of different ecological groups will be included in genome sequencing. Available genome and corresponding protein sequence techniques should enable identification of proteins that are uniquely induced in response to stress.

Photoresponses and circadian rhythm

Because fungi lack chlorophyll, the tendency has been to disregard the effect of light on fungal development. The Nobel laureate Max Delbruck left his highly successful

phage research and was drawn to phototropic curvature of sporangiophore of *Phycomyces blakesleeanus*. Currently, the effects of light on *Neurospora* are being intensively studied. When grown in a growth medium in a race tube (Figure 5), an alternating pattern of hyphae and asexual spores (conidia) are produced once every 22 h – a manifestation of an endogenous time-keeping system. Several mutants show altered period lengths (16–29 h) or arrhythmicity, suggesting that genes affect the operation of the circadian clock[70]. For example, one mutant has a period of ~19 h, another has a period of ~22 h, and another is arrhythmic. These mutants are alleles of the *frequency* gene, whose product contributes to a molecular oscillator whose rate of degradation is a major determining factor for the period length of the circadian clock. At present, the model of circadian rhythm in this fungus (Figure 6) envisages transcription of *frq* gene(s), followed by production of FRQ protein(s), their feedback on self-transcription, degradation of FRQ protein(s) releasing the negative feedback, allowing a new round of transcription and resulting in molecular oscillations of RNA and protein. The relative levels of frq mRNA and FRQ protein levels cycle with a 22-h period in the wild-type strain grown in constant darkness. It is therefore the oscillator determining the conidiation rhythm**.** Among important research goals is the identification of genes regulated by *frq* and the signalling pathways from the environment through which the cellular clock is synchronized to the external world.

It seems likely that as knowledge of this phenomenon and methods to determine this becomes known, more fungi will be found providing insight into the nature and possible ecological role of this phenomenon. Many principles of light input to circadian clocks that are found in *Neurospora* also apply to higher eukaryotes, such as plants, insects and mammals.

Decomposition of biomass

Since cellulose – the main constituent of biomass – is insoluble, its decomposition was simply viewed as a problem of converting it into soluble sugars by extracellularly secreted enzymes for uptake – a process which could be translated for bioconversion of cellulosic material into glucose and ethanol. In the 1970s, a world-wide programme was started for screening and selecting fungi which secreted mixtures of *b*-1,4 (exo- and endo) glucanases and *b*-glucosidase – the three primary enzymes thought to cooperate in complete cellulose hydrolysis. The US Army Laboratory at Natick claimed having developed strains of Trichoderma that secreted up to 30 g cellulase enzyme per litre of the culture medium, generating much euphoria for large-scale conversion of cellulosic material for manufacturing ethanol. However, even before it was understood how fungi degrade cellulosic material in nature, basic questions were brushed aside in favour of practical ends. Culture filtrates of the fungus which degraded cellulose completely in culture flasks, had limited action on cellulose under *in vitro* conditions[71]. What had been overlooked is that cellulose degradation is intimately associated with growth, an idea reiterated by work done in Wessels' laboratory[72]. Other factors appear to be involved, such as the adherence of fungal hypha to substrate by mucilage (a glucan sheath), and the synergistic action of enzymes aggregated on cell surface as multienzyme complexes termed 'cellulosome'[73], which is disaggregated as autolysis of cell wall sets in upon consumption of cellulose. This possibility is suggested by the finding of cellulosomal cellulases in cellulolytic bacteria and the observation that polymer (lignin) degrading activity is associated with the mucilage (glucan) sheath. Whether cellulose degradation by highly efficient fungi also occurs primarily through synergistic action of

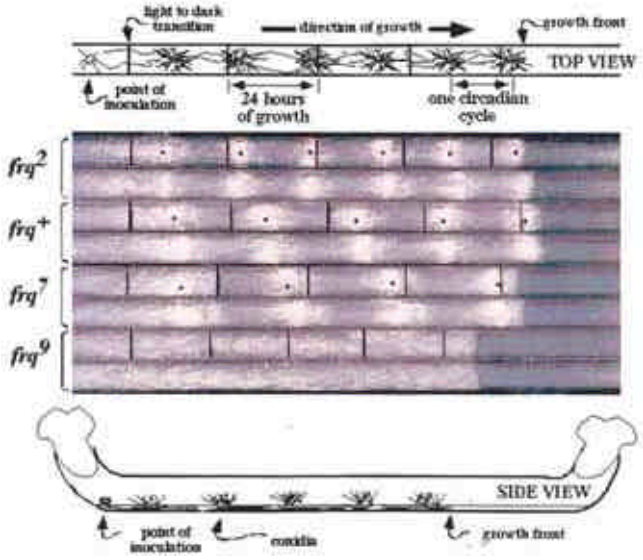


Figure 5. Conidiation in *N. crassa* – A model of investigation on biological rhythms. Photo courtesy: Jennifer Loros, Dartmouth Medical School, Hanover, USA.

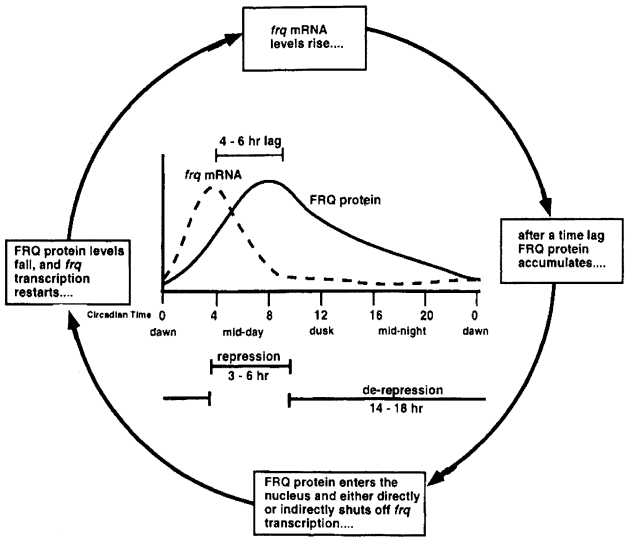


Figure 6. Model of circadian rhythm in *N. crassa*. Reproduced from Bell-Pedersen *et al.*[70], © Indian Academy of Sciences, by permission.

enzymes aggregated on cell surface as multienzyme complexes termed 'cellulosome', needs to be examined[74]. An 'old' hypothesis that wood decay fungi employ extracellular reactive oxygen species and oxidoreductase enzymes to cleave lignocellulose is being revived[75]. Few studies have compared the rates of biomass decomposition by pure cultures with those with mixed cultures.

Fungal populations

The study of population biology is based on field observations and collections together with experiments in the laboratory, and embraces many fundamental biological issues, for example: How many species does it comprise of? In what type of habitats and climates do they occur? How different are their life cycles? What types of variations occur among individuals in a population? How can the genetic variation be used to chart the course both of evolution and speciation? A fungus which has emerged extremely suitable for resolving these questions is *Neurospora*, collected globally by David Perkins[76], and over 4000 cultures derived from nature made freely available to investigators. Species-specific tester strains have been developed, making it rather simple to assign them to species based on crossing and production of ascospores. It has revealed the common occurrence of mitochondrial plasmids and a question that has emerged is how homologous plasmids, including senescence-inducing plasmids have become distributed across continents? The strains collected from different latitudes are beginning to be used to examine if the period lengths of the circadian cycle is an adaptation to length of day and night. Some type of variants obtained from collections in nature would have been difficult, if not impossible, to produce in the laboratory. For example, the spore killer[77] or microcyclic strains[78] were discovered. In the microcycle strain, a germinating conidium directly forms a conidiophore, totally bypassing the intervening mycelium phase which produces conidiophores. The discovery of microcycle strains suggested that a master gene controls the expression of a large number of conidiation genes. Conditions that activate the master gene result in precocious asexual reproduction.

A question central to population biology is why certain fungi are ubiquitous, but some closely related forms are restricted to special habits? For example, although global collections of *N. intermedia* strains are largely orange or pink-orange coloured, a yellow type is almost exclusively found on roasted corn cobs after the kernels have been eaten and the cobs discarded. The yellow *Neurospora* is distinctive not only in its habitat, but also in its conidia size and nuclear number of its conidia. There is no evidence that because of the geographical isolation, the orange and yellow *N. intermedia* are members of an interbreeding population. The phylogenetic trees constructed based on variation in the non-transcribed spacer suggested that the yellow isolates are a separate lineage, distinct from a larger *N. crassa*/*N. intermedia* clade. Although the yellow type can be coerced to mate with the orange type, it is doubtful if this occurs in nature. Rather, the yellow type has diverged morphologically, ecologically and phylogenetically[79] and is on the threshold of evolving into a distinct species. Fungi are excellent material for study of process of speciation by physical, temporal and reproductive isolation.

Biotechnology

The ability of certain fungal species to secrete large amounts of proteins into the culture medium has generated the prospects of their use for large-scale production of native and heterologous proteins. With secreted proteins the recovery of protein is easier, as there are no tough cell walls to break. A revelation is that though extensively branched and possessing a large surface area, the mycelium secretes protein *only* through the hyphal tips[72]. As each branch has a tip of its own, this suggests that the amount of protein secreted may depend on the intensity of branching. Consequently, research is required to determine whether the degree of branching can be increased by chemical or genetical methods concomitant with increased secretion of protein. The availability of genome sequence information and gene arrays can provide a new opportunity to investigate the protein secretion process. As many post-translational modifications of proteins (glycosylation, proteolytic processing and disulphide formation) occur in eukaryotic systems, understanding the control of these processes and the factors required for the transport of protein from the endoplasmic reticulum and Golgi, and delivery of secretory vesicles to the hyphal tip are important to improve the stability, quality and yield of the protein. Transgenic fungi offer themselves not only for the production of enzymes of industrial use but also for vaccines, and human therapeutic proteins such as growth factors, cytokines, and protein hormones (http://www.bio.mq.edu.au/dept/centres/edge/fungalbt.html, www.genengnews.com).

Comparative genomics

The first eukaryotic genome to be sequenced was that of yeast[80]. The genome sequence of *Neurospora*[81] was released in 2003. Whereas yeast is a unicellular fungus, *Neurospora* is multicellular, having at least 28 morphologically different cell types[82]. Consistent with the greater biological complexity, *Neurospora* possesses nearly twice (10,082) as many genes as *S. cerevisiae* (6300). *Neurospora* encodes approximately 25% more transporter systems than does *S. cerevisiae*. In sharp contrast to the cell wall of *Neurospora*, yeast lacks (1, 6) *b*-linked glucans. However, the presence of chitin and its absence in plants and animals indicates that anti-chitin compounds could be targets for development of anti-fungal compounds. Furthermore, though a saprophyte, *Neurospora* possesses genes for enzymes

which digest plant cell wall required for fungal pathogenesis. Comparisons of genomes of the saprophytic (e.g. *N. crassa, A. nidulans*) and pathogenic fungi (e.g. *M. grisea, U. maydis*) should identify genes specifically found in pathogenic fungi for development of antifungal drugs and fungicides. In contrast to yeast, *Neurospora* can methylate its own DNA to silence (inactivate) genes. A surprise revelation was that *N. crassa* has homologues of phytochrome required for sensing red–far-red sensing in plants. The fungus shares genes with complex organisms that measure time (biological clock).

Future challenges

The past progress in fungal biology has been impressive. It has opened up many penetrating questions for the future. For example: How is a hypha shaped as a tube of constant diameter? How are the sites of hyphal branching determined? How do hyphal tips act as a strong sink for nutrients? Can the degree of branching be increased so that a desired protein is secreted out in increased amounts? How do the single-celled yeasts mark the sites for positioning a new bud? How are the nuclear-encoded proteins synthesized in the cytoplasm, targetted into an organelle, such as the mitochondrion? How do organelles move and position themselves in the hypha? Of what significance is the multinuclear condition of the hypha? What determines competition or cooperation among different nuclear or mitochondrial genomes? What signals are exchanged between a photosynthetic plant and a mycorrhizal fungus before they can enter into a symbiotic relationship? How does a parasitic fungus find an entry point in a plant leaf by 'touch'? What weapons does the fungus use to breach host defence mechanisms? How does a fungus form a spore-bearing structure of symmetry, like the flower of a plant, and produce prodigious numbers of conidia with great economy of space? What timing device does a fungus have for discharging spores at the most propitious time, enhancing their survival, dissemination and germination? How does a fungus decay wood of enormous strength? How do fungi (mushroom, polypore or bracket fungus) form large three-dimensional fruiting bodies in the absence of longitudinal division? Of what significance is the production of multiple types of spore by an individual fungus? How do fungi mate in the absence of morphological differentiation of the mating partners? How do fungi protect themselves from attack by homologous or heterologous DNA molecules in their surroundings? How do mesophilic and thermophilic fungi, though adapted to widely different temperatures, maintain relatively similar metabolic rates? Once these issues are resolved, several more, unpredictable ones will undoubtedly arise.

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