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A study of genetic characterization and fungus diversity

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Abstract
The extent of fungal diversity is reviewed, with respect to revised estimates of the numbers of plant species, and recent data on the extent of novelty in tropical forests, unexplored habitats, and numbers of orphaned, cryptic, and collected but yet undescribed species. Collections of fungal cultures are considered to be better referred to as "genetic resource collections" rather than "culture collections" to mesh with current terminology in other groups of organisms. The extent of holdings relative to the numbers of known and estimated species are reviewed and compared with those of vascular plants in botanic gardens and seed banks. The role of collections in supporting fungal genomics and molecular biology, and as a source of vouchers to vindicate published work in all aspects of mycology, is highlighted. Information is presented on the extent to which collections worldwide document and conserves the Earth's fungal genetic resource. Finally, the special role and responsibilities of CBS, as the major centre for the conservation of fungal genetic resources worldwide, is emphasized.
Over the last 10 years plant pathologists have begun to realize that more knowledge about the genetic structure of populations of plant pathogens is needed to implement effective control strategies. Research on the genetic structure of fungal populations has mushroomed, and review studies that summarize these studies are numerous. Although the number of fungal studies has increased greatly, the most comprehensive work has focused on a small number of plant-pathogenic fungi. The majority of these fungi can be recognized easily by their fruiting bodies or disease symptoms on aboveground plant parts. It has proven more difficult to assess the genetic structure of fungal populations that exist mainly belowground, because the distribution of individuals cannot be visualized directly and appropriate sampling procedures are less obvious and more cumbersome. Nevertheless, substantial progress has been made in interpreting the population genetic structure of some soilborne fungi. The purpose of this study is to provide an overview of the tools and techniques of fungal population genetics.

Keywords: genetic, characterization, fungus diversity, fungi, progress, populations, plant

Introduction
The issue of Fungus diversity, its extent and conservation, has attracted more attention in the last 10–15 years than in any period of history. But what implications do the recent debates have for collections of Fungus cultures, especially in the genomic age? The centenary of the Centraalbureau voor Schimmel cultures (CBS), the Fungus Biodiversity Centre, now in Utrecht, provides an appropriate occasion to consider the emerging issues, the extent of the problems, and implications for the role of such collections [1]. Just as living organisms evolve to meet environmental challenges, so the scientific infrastructure needs to adapt. In particular, institutions must both meet the immediate needs of successive generations of scientists, and also position themselves to be able to fulfil anticipated future demands. Having been privileged to be entrusted with the management of one of the world's leading mycological centres for 14 years, through a period of major change and relocation [2-4], I am acutely aware of the need for pragmatic approaches. Here the current state of our knowledge of Fungus diversity, existing Fungus genetic resource collections, and the challenges collections face in supporting the needs of Fungus genomics and molecular biology, as well as those of conservation [5].
Soil microcosms and DNA extraction to study the Fungus community in wheat rhizosphere soil, we collected soil from a small field plot on the campus of the University of Utrecht located on the Uithof, Utrecht, and The Netherlands. This is a clay soil containing 4% organic matter with a pH of 5.0. Samples from this soil were used for plating culturable fungi and for setting up the microcosm experiment [6-8]. The soil was air dried and sieved, and nine small pots (diameter, 10 cm) were filled. Soil was seeded with eight seeds of Triticum aestivum cv. Baldus per pot. Fluctuations in moisture content were minimized by supplying water daily to keep the soil moisture content at 20%. Microcosms were incubated in a climate chamber with a light-dark regimen of 16 and 8 h at 20 and 15°C, respectively. Microcosms were sampled in duplicate on days 5 and 10. Bulk soil samples of 3g were taken from root-free soil. Rhizosphere soil was obtained by gently shaking the soil from the roots, and roots with adhering soil were added to 50-ml polypropylene tubes with 10 ml of sterile sodium phosphate buffer (120 mM; pH 8) and 1g of gravel. Tubes were vortexes for 30 s, and the buffer-soil slurry mixture was poured into a new tube, leaving the gravel and roots behind [9, 10]. Total DNA was extracted from the rhizosphere soil slurry by using a bead beater. One microliter of the extract was used for PCR amplification.

Literature Review

This research covers basic and contemporary systematic principles and methods as applied to vascular plants, including classification, identification skills, phylogenetics, molecular approaches, and surveys of important families of major groups of flowering plants via lectures and lab practice. Students are expected to achieve the following objectives after successfully completing the course: describe a plant using botanical terms, identify a plant using the key mechanics, name and publish a new species, recognize large and common families of flowering plants, interpret plant relationships depicted on phylogenetic trees with proper terms, exhibit basic knowledge in molecular approaches applied to systematics, demonstrate knowledge in the current understanding of angiosperm phylogeny and evolution. PB 503 students will carry out a term project in molecular systematics involving project design, data acquiring from Genbank, and phylogenetic analyses.

Evaluation of students will be based on exams (4 on lecture and 3 on lab), collection project, field trips, and term paper (for PB 503 students). This Research in plant geography that emphasizes what grows where and why. Historical and ecological approaches are emphasized, and discussions include climatological, pedological, historical, paleobotanical, and more recent approached to understanding the present distribution of plants. A field trip to Green Swamp and Jones Lake to see some more unusual ecosystems is included. Course covers the structure and function of plant communities, with emphasis on both classical and recent research. Through a lecture/laboratory format, the course introduces students to four major areas of emphasis in plant community ecology. The first of these focuses on definition of the plant community, sampling approaches, measurement and description of community properties, and analysis of community data, with coverage of gradient analysis and classification. Lectures on vegetation of the world and vegetation of North Carolina are included. The second area of emphasis is the influence of environmental characteristics (particularly climate, topography, substrate and soils, and fire) on the nature and spatial distribution of communities. The third area of emphasis is community dynamics, with coverage of disturbance, succession, and modeling of vegetation change through time.

Lectures on plant geography and paleobotany are included. The fourth area of emphasis is community organization and function, with coverage of modern theoretical treatments and their predictions; much of this last portion of the course focuses on patterns and processes related to the diversity of species in the plant community. The laboratory is field-oriented and introduces students to natural communities of Wake County (eastern North Carolina Piedmont) and their distribution across the landscape. An introduction to methods of inventory for vegetation and environment is included. In addition to the regular weekly laboratory meetings, there are also two extended field laboratories (three or four days each), one to the mountains, and the other to the coastal plain and coastal fringe. Participation in at least one of these longer laboratories is mandatory. Expanding population growth near lakes, rivers, estuaries, and coastal oceans is effectively shrinking the quality and quantity of freshwater and marine resources worldwide. A general understanding of the scientific basis of impacts from nutrient pollution, toxic chemicals, acidification, global warming, overfishing, and related stresses, and the overarching policy/political controls, is critically needed to restore and optimally manage these systems, and to protect the health of humans who depend upon them for potable water supplies and fish resources.

Fungus

A fungus is any member of a large group of eukaryotic organisms that includes microorganisms such as yeasts and molds, as well as the more familiar mushrooms. Once considered to be plants, the Fungi are now classified as a kingdom separate from plants and animals. A major difference is that fungal cells have cell walls that contain chitin, unlike the cell walls of plants, which contain cellulose. These and other differences show that the fungi form a single group of related organisms, named the Eumycota (true fungi or Eumycetes), that share a common ancestor (a monophyletic group). This fungal group is distinct from the structurally similar slime molds (myxomycetes) and water molds (oomycetes). The discipline of biology devoted to the study of fungi is known as mycology, which is often regarded as a branch of botany, even though genetic studies have shown that fungi are more closely related to animals than to plants. Fungi reproduce via spores, which are often produced on specialized structures or in fruiting bodies, such as the head of a mushroom.

Unique Features

- Some species grow as single-celled yeasts that reproduce by budding or binary fission. Dimorphic fungi can switch between a yeast phase and a hyphal phase in response to environmental conditions.
- The fungal cell wall is composed of glucans and chitin; while the former compounds are also found in plants and the latter in the exoskeleton of arthropods, fungi are the only organisms that combine these two structural molecules in their cell wall. In contrast to plants and the oomycetes, fungal cell walls do not contain cellulose.

Fig 1: Omphalotus Nidiformis, a Bioluminescent Mushroom

Most fungi lack an efficient system for long-distance transport of water and nutrients, such as the xylem and phloem in many plants. To overcome these limitations, some fungi, such as

Armillaria, form rhizomorphs [21], that resemble and perform functions similar to the roots of plants.

Diversity

Fungi have a worldwide distribution, and grow in a wide range of habitats, including extreme environments such as deserts or areas with high salt concentrations or ionizing radiation, as well as in deep sea sediments [28]. Some can survive the intense UV and cosmic radiation encountered during space travel. Most grow in terrestrial environments, but several species live partly or solely in aquatic habitats, such as the chytrid fungus Batrachochytrium dendrobatidis, which has been responsible for a worldwide decline in amphibian populations. This organism spends part of its life cycle as a motile zoospore, enabling it to propel itself through water and enter its amphibian host. Other examples include fungi living in hydrothermal areas of the ocean.

Along with bacteria, fungi are the primary decomposers of organic matter in most if not all terrestrial ecosystems worldwide. Around 100,000 species have been formally described by taxonomists, but the true dimension of global fungal diversity is not well understood. Based on observations of the ratio of the number of fungal species to the number of plant species in selected environments, the fungal kingdom has been estimated to contain about 1.5 million species. In mycology, species have historically been distinguished using a variety of species concepts. Classification based on morphological characteristics, such as the size and shape of spores or fruiting structures, has traditionally dominated fungal taxonomy. Species may also be distinguished by their biochemical and physiological characteristics, such as their ability to metabolize certain biochemicals, or their reaction to chemical tests. The biological species concept discriminates species based on their ability to mate. The application of molecular tools, such as DNA sequencing and phylogenetic analysis, to study diversity has greatly enhanced the resolution and added robustness to estimates of genetic diversity within various taxonomic groups.

Fungal Strains, Culture Conditions, and Amplification Range

Various fungal species from all major taxa were used to test the primer sets. Lyophilized cultures were rehydrated with a sterile 0.85% NaCl solution and applied to potato dextrose agar plates. Plates were incubated at 28°C for several days to 2 weeks, until sufficient hyphal growth was observed.

Fungi were also cultured from the soil. Samples of 10 g of soil were shaken in 100 ml of a $MgSO_4$ solution (10 mM) for 15 min. Serial dilutions were plated onto 2% malt extract agar containing 0.33% Solacol and 200 ppm of aureomycine. Plates were incubated at 20°C for 1 week. Fungi with different morphologies were then selected and streaked onto cornmeal agar. The plates were incubated for 1 to 2 weeks, and some of the fungi were identified by W. Gams at the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands.

DNA was isolated by scraping hyphae from the agar surface. Cells were disrupted, DNA was released by bead beating, and the resulting lysate was purified as described by Smit *et al*. Prior to the examination of the amplification range of the primer sets EF4-EF3, EF4-fung5, and EF4-NS3-GC on a collection of fungal isolates, fungal cell lysis and the quality of the extracted DNA were tested by using the general eukaryotic primer set 106-107. Only 1 strain (Coniothyrium sporulosum) out of 27 was poorly lysed (results not shown). A positive signal obtained by using these primers indicated that the fungal cells were lysed by the bead-beating method and that the quality of the DNA was sufficiently high for PCR amplification.

The Extent of Fungal Diversity

Since the number of fungi present on Earth was conservatively estimated at 1.5 million species, alternative estimates ranging from 0.5– 9.9 million have been published by other authors. Schmitt & Mueller (2004) calculated that there must be a minimum of 0.6 million species, using criteria that most mycologists would regard as excessively conservative to establish a lower boundary figure. In addition, a major comparative metadata analysis of macrofungi and plant diversity has been undertaken for the first time. Data from 25 studies in different parts of Asia, Europe and North America were analyzed statistically by Schmitt *et al*. (2004); the results showed that fungal species richness was much higher than that of the plants, demonstrated that tree species diversity was a good predictor of macrofungal diversity, supported the use of ratio estimates to measure fungal species richness, and were consistent with the high estimates of species numbers made by Hawksworth (1991).

Extrapolations made from the numbers of fungi and plants growing in particularly well-studied countries to the global scale are heavily influenced by the number of plant species recognized. Hawksworth (1991) used a figure of 270 000 plants worldwide. Since then larger estimates have been made, which would imply that the extrapolated numbers of fungi could be too low. For example, Prance *et al*. (2000) estimated 300–320 000 plant species, taking note of those that remained to be described from the tropics, while Govaerts (2001) argued that there were probably already 420 000 accepted and known seed plant species, based on the number of published scientific names and synonymy rates. However, using results from selected monographs, Scotland & Wortley (2003) considered Govaert's estimate had used too low a synonymy rate and that his figure could be an overestimate by more than 200 000 species. Developing this approach further, Wortley & Scotland (2004) extrapolated from synonymy rates in 17 monographs (mean 66 %, i.e. about two in three of the published names are synonyms) that, with 95 % confidence, the number was in the range 117 734–575 320. These last authors did not provide a revised estimate, however, recognizing the need to analyse many more monographs. What is evident from these debates is that the 270 000 figure used in my original extrapolations would be regarded as low by many plant taxonomists who have subsequently addressed this question, with Raven (2004) settling on 300 000. This is a further reason for retaining the 1.5 million species figure as the current working hypothesis, which remains widely accepted by mycologists.

Fungal Genetic Resource Collections

Institutions or activities must reconsider and reinterpret their objectives in the language of the day; failure so to do may

endanger their survival. The single act of re-labelling "culture collections" as "genetic re- source collections" would immediately make the link with agendas of the 158 governments who have so far ratified the Convention on Biological Diversity, and overview assessments of global genetic resources. The Culture Collection of the then International Mycological Institute (now part of CABI Bioscience) made the name-change to Genetic Resource Collection in 1992.

The label "culture collections" is a barrier to communication. It has hindered the forging of links with the rise of interest and funding devoted to the exsitu conservation of plants and animals during the last two decades. Interdisciplinary meetings on "genetic resources" have only exceptionally considered microbial groups, and it is proving a slow process to change the perception that fungi and microorganisms are something apart from "genetic resources".

In addition to the value of using the phrase "genetic resource collections" with respect to the Convention on Biological Diversity, it would also make a second natural link: to the concept of "biological resource centres", on which recommendations for actions by governments have been made by the countries of the Organization for Economic Cooperation and Development.

Documenting and Conserving the Fungal Genetic Resources

The World Data Center for Microrganisms currently holds data on 370251 strains of fungi. The number of names to which these are assigned is about 24000. No critical revision of the names to eliminate synonyms and teleomorph / anamorph duplications has been carried out. However, when an analysis of the holdings was last carried out, the proportion of strains held to checked and accepted species names was 22:1. Assuming that this overall ratio applies to the current holdings, this suggests that around 16830 species may really be represented. While this is a significant increase on the 11500 species represented in the last analysis, and constitutes about 30.5 % of the estimated culturable species, it amounts to only 16% of the estimated already known 100 000 species of fungi, and 1.1% of the estimated 1.5 million on Earth. It is salutary to reflect that these last two figures were 17% and 0.8% respectively in the last analysis. This implies that, collectively, the world's fungal culture collections are scarcely even keeping abreast of the new species continually being discovered, l*et al*one making significant inroads into conserving a substantially greater proportion even of the known fungi.

In the case of flowering plants, there is an amazing 6 million accessions of plant genetic resources worldwide, although around half relate to major crops; 90% of these accessions are in seed banks, with 85000 species in cultivation, primarily in botanic gardens. This implies that about 28% of the estimated 300000 known plant species are secured in genetic resource collections. Against this benchmark, the 16% of known fungi safeguarded is commendable, bearing in mind the disparity between resources devoted to botanic gardens and seed banks and those available for fungal collections. For example, there are about 2000 botanic gardens dispersed through 148 countries, compared with 483 fungal genetic resource collections spread through only 61 (Hideaki Sugawara, pers. comm.). It has been estimated that as many as half of the world's plant species may qualify as threatened with extinction under the criteria used by IUCN-The World Conservation Union, and botanic gardens and seed banks now focus on endangered species. Knowledge of the distribution of most fungal groups is too poor to enable endangered fungi to be targeted in the same way, with the exception of some macrolichens and hydnaceous fungi. Because so many fungi are obligate associates of particular plants, it may well be that more than half of the Earth's fungi are also entering endangered categories. In order to safeguard the global fungal genetic resource for posterity, collections consequently need to endeavour to secure material of as many different species as possible in a viable state. If this is not treated as a matter of urgency, there may be no second chance.

With so many fungi that can be grown in culture still to preserve, the incorporation into collections of living material that cannot be (or at least has not yet been) grown has tended to take a back seat. However, there are increasing numbers of studies that show that cryopreservation of fungal-infected host tissues is effective, for example with rust fungi. This is an area where there is immense scope for fungal genetic resource collections to undertake basic research, with a view to developing protocols that can be widely used to conserve "unculturable" or "recalcitrant" fungi.

Morphology Microscopic Structures

A hypha of Hyaloperonospora parasitica (downy mildew) growing within the leaf tissue of Arabidopsis thaliana. The long structure is the hypha, and the little spheres are haustoria, which extract nutrients from the plant cells.

Most fungi grow as hyphae, which are cylindrical, thread-like structures 2–10 µm in diameter and up to several centimeters in length. Hyphae grow at their tips (apices); new tips typically form by branching from sub-apical hyphal locations or occasionally by bifurcation (forking) of growing tips [37]. The combination of apical growth and branching/forking leads to the development of a mycelium, an interconnected network of hyphae. Hyphae can be septate, i.e., divided into compartments separated by cross walls (septa), each compartment containing one or more nuclei, or can be coenocytic, i.e., lacking hyphal compartmentalization. Septa have pores, such as the dolipore septa in the basidiomycetes that allow cytoplasm, organelles, and sometimes nuclei to pass through. Coenocytic hyphae are essentially multinucleate supercells. Many species have developed specialized hyphal structures for nutrient uptake from living hosts; examples include haustoria in plant parasites of most phyla, and arbuscules of several mycorrhizal fungi, which penetrate into the host cells to consume nutrients.

Although fungi are part of the opisthokont clade—a grouping of evolutionarily related organisms broadly characterized by a single posterior flagellum—all phyla except for the chytrids have lost their posterior flagella [43]. Fungi are unusual among the eukaryotes in having a cell wall that, besides glucans and other typical components, contains the biopolymer chitin.

Macroscopic Structures

Fig 2: Armillaria Ostoyae

Fungal mycelia can become visible macroscopically, for example, as concentric rings on various surfaces, such as damp walls, and on other substrates, such as spoilt food, and are commonly and generically called mold (British spelling, mould); mycelia grown on solid agar media in laboratory petri dishes are usually referred to as colonies, exhibiting characteristic macroscopic growth shapes and colors, due to spores or pigmentation. Some individual fungal colonies can grow to a very large size and mass, in some cases reaching extraordinary dimensions and ages as in the case of a clonal colony of Armillaria ostoyae, which extends over an area of more than 900 ha, with an estimated age of nearly 9,000 years. In the ascomycetes, a specialized structure important in sexual reproduction is the apothecium, a cup-shaped structure that holds the hymenium, a layer of tissue containing the spore-bearing cells [47]. The fruiting bodies of the basidiomycetes and some ascomycetes can sometimes grow very large, and are well-known as mushrooms.

Growth and Physiology

The growth of fungi as filamentous hyphae on or in solid substrates or as single cells in aquatic environments is adapted for the efficient extraction of nutrients, because these growth forms have high surface area to volume ratios. Hyphae are specifically adapted for growth on solid surfaces, and to invade substrates and tissues [49]. They can exert large penetrative mechanical forces; for example, the plant pathogen Magnapor the grisea forms a structure called an appressorium which evolved to puncture plant tissues [50]. The pressure generated by the appressorium, directed against the plant epidermis, can exceed 8 MPa (80 bars). The filamentous fungus Paecilomyces lilacinus uses a similar structure to penetrate the eggs of plant-parasitic nematodes.

Fig 3

Mold covering a decaying peach. The frames were taken approximately 12 hours apart over a period of six days.

The mechanical pressure exerted by the appressorium is generated from physiological processes that increase intracellular turgor by producing osmolytes such as glycerol. Morphological adaptations such as these are complemented by hydrolytic enzymes secreted into the environment to digest large organic molecules—such as polysaccharides, proteins, lipids, and other organic substrates—into smaller molecules that may then be absorbed as nutrients. While the vast majority of filamentous (hypha-forming) fungi grow in a polar or directional fashion by extension at the tip of the hypha [56], intercalary extension as in the case of some endophytic fungi [57], or by volume expansion during the development of mushroom stipes and other large organs [58] are alternative forms of growth. Growth of fungi as multicellular structures consisting of somatic and reproductive cells—as seen and independently evolved in animals and plants has several functions, including the development of fruiting bodies for dissemination of sexual spores and biofilms for substrate colonization and intercellular communication.

Reproduction

Fig 4: Polyporus squamosus

Fungal reproduction is complex, reflecting the heterogeneity in lifestyles and genetic makeup within this Kingdom of organisms. It is estimated that a third of all fungi use more than one type of reproduction, frequently in two well-differentiated life cycle stages (the teleomorph and the anamorph). Environmental conditions trigger genetically determined developmental programs that lead to the creation of specialized structures for sexual or asexual reproduction. These structures aid both reproduction and efficient dispersal of spores or spore-containing propagules.

Asexual Reproduction

Asexual reproduction via vegetative spores or through mycelial fragmentation is common; it maintains clonal populations adapted to a specific niche, and allows more rapid dispersal than sexual reproduction [66]. In the case of the "Fungi imperfecti" or Deuteromycota, which lack a sexual cycle, it is the only means of propagation.

Conclusion

Fungus classification is far from static, and even which organisms are actually members of Fungi is changing. For example, the group Trichomycetes describes gut inhabitants of

arthropods that share similarities with zygomycetes. Molecular phylogenetic studies have demonstrated that two of the four orders of Trichomycetes are actually members of the Mesomycetozoea protest group. Other organisms that were previously considered to be Fungi because of their heterotrophic, mold-like growth forms are now classified as stramenopiles (Oomycota, Hyphochytriomycota, and Labyrinthulomycota) or slime molds (Myxomycota, Plasmodiomycota, Dictyosteliomycota, Acrasiomycota). More interesting for mycologists are the findings that some species previously considered protozoa are actually Fungi. The most revolutionary addition to the Fungus lineage has occurred with phylogenetic evidence indicating the protist group microsporidia is closely related to Fungi–possibly derived from zygomycetes or sister to the genus *Rozella* on the earliest branch in the Fungus nation. Microsporidia are highly specialized intracellular parasites (primarily of animals) that lack mitochondria but have chitin and trehalose in their spores (similar to Fungi). All molecular studies have shown that microsporidia evolve at an extremely accelerated rate of evolution, making their placement in the Tree of Life difficult. The relationship with fungi is supported by many single and multiple gene phylogenies, but an exact placement within the fungi has not received strong support.

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