

Pandora's Mycological Box: Molecular sequences vs. morphology in understanding fungal relationships and biodiversity

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Summary

Fundamental reappraisals of diverse traditional ideas in mycology have become necessary as a result of molecular insights. These different insights are discussed in relation to: the positions of microsporidia, slime moulds and oomycetes; the basal position of lichen fungi in the evolution of ascomycetes forming fruit bodies; remodelling of orders and families; changed generic concepts; the issue of whether permitting a dual nomenclature for the different states of pleomorphic fungi should be continued; and the recognition of additional cryptic species within a "species". The molecular data has necessitated a reassessment of the systematic importance of many types of characters. Also, the techniques open exciting horizons and undreamed of abilities through being able to identify non-sporing fungi in ecological samples and plant material, and revealing unexpected levels of diversity in hitherto little-explored habitats. Major advances in understanding how fungi operate through total genomic approaches can be anticipated as more are completely sequenced. The Pandora's box of molecular surprises is to be seen as one of blessings and not one of miseries and evils.

Key words

Cryptic species, Fruit bodies, Fungi, Genomics, Lichens, Phylogeny, Systematics

Delving into molecular phylogenetics with fungi is like opening the mythical box of Pandora, the first woman in Greek mythology. The box contained either all manner of miseries and evils, or according to later versions of the myth, blessings. The contents were unknown until released, but then the miseries or blessings flew rapidly all over the Earth, and there was no escape from their impact. And there is no escape for mycologists from the impact of molecular insights.

The title of a news item reporting a British Mycological Society and British Society for Medical Mycology debate held in Manchester in March 2003, read 'Molecules vs Morphology' [77]. I know that some traditional mycologists see the move towards molecular approaches as a threat, but others have enthusiastically embraced

them, either learning the techniques themselves or collaborating in teams.

Fundamental reappraisals of traditional ideas have become necessary, from the level of determining what organisms belong in the *Fungi*, through reorganizing systems of orders and families, to the remodelling of genera, and the realization that many fungal "species" are several and not one. And new vistas of research possibilities have opened, yielding fascinating information on fungal diversity and the importance of fungi in ecosystems. Here, I touch on a number of aspects where molecular approaches have had significant impacts with respect to our understanding of relationships and biodiversity. The examples are necessarily eclectic, many drawn from studies in which I have been involved, but have been selected to be indicative of the new insights being obtained into different types of situations.

What organisms are fungi?

The idea that fungi were fundamentally different from plants and animals has its roots in the mid-nineteenth century, but the idea that they constituted a separate kingdom only started to be generally accepted following the seminal paper of Whittaker [78]. Molecular work on DNA and proteins has now made it clear that the kingdom *Fungi* is more closely allied to that of animals *Animalia*, than to the plant kingdom *Viridiplantae* [11,63,74].

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But at the same time it has become clear that the slime-moulds, although traditionally studied by mycologists, are indeed really protozoa, and that the oomycete fungi, including such familiar genera as *Phytophthora* and *Pythium*, are most closely allied to some algal groups and quite outside the kingdom *Fungi*; these are now placed along with photosynthetic relatives in the kingdom *Straminipila*¹ [18]. In addition, various organisms not traditionally studied by mycologists have proved by molecular studies to belong in the *Fungi*. These include anaerobic chytridiaceous fungi in animal rumens, some trichomyces in insect guts, and *Pneumocystis*; this last genus was long considered a protozoan and contains species causing fatal pneumonia's in immunocompromised humans. More recently, it has now been established that the microsporidians, also previously interpreted as protozoa and with around 1,000 species, belong amongst the zygomycetes [39,42]. Fascinatingly, both the anaerobic chytrids and microsporidia have lost their mitochondrial genomes [10].

In view of these considerations, it is important to distinguish between "fungi" (organisms studied by mycologists) and "*Fungi*" (members of the kingdom *Fungi*). In this presentation I use "fungi" to mean all organisms studied by mycologists, including lichen-fungi, microsporidia, moulds, mushrooms, straminipiles, and yeasts.

Turmoil in classes, orders and families

Molecular phylogenetic studies have led to a decade of instability in the hierarchical classification of fungi. This was inevitable as into the late 1990s rather a small proportion of the families and genera had any representatives sequenced, and those that, quite understandably, were largely ones which grew in culture or which were of economic or medical interest. As sequencing became more routine, this situation has changed dramatically, and there are currently³ sequences from 14,054 differently named fungal species in GenBank; allowing for synonyms and separately named anamorphs [33], that amounts to around 13% of the currently known 100,000 species.

Most dramatic has been the remodelling of orders and families in the larger basidiomycetes, where 14 main clades are now recognized [2]. The greatest surprise to traditional systems of classification is that it is now incontestable that whether fruit bodies are stalked and mushroom-like, unstalked but with caps, coral-like, closed (truffle-like), bracket-like, or paint-like or resupinate, and whether they have pores, gills, teeth or a smooth hymenium, are much less important indicators of evolutionary relationships than more fundamental characters of tissue types and the nature of the basidiospores. In consequence, fungi that would have never been considered as close in the 1980s, now prove to be bedfellows [38]. Examples are: *Auriscalpium*, *Hericium*, *Peniophora*, *Russula*, and *Stereum* (all in the russuloid clade); and *Botryobasidium*, *Cantharellus*, *Clavulina*, *Thanatephorus*, and *Tulasnella* (all in the cantharelloid clade). Within some of these main clades, substantial numbers of subclades are distinguishable, for example, 117 within the euagarics clade [56]. Former familiar classes such as *Gasteromycetes* have passed

into oblivion, being widely dispersed amongst different clades.

Through the mid-1980s until the late 1990s, categories above the rank of order were not used in some ascomycete systems commended for general use. For example, Eriksson & Hawksworth [26] recognized 44 orders, but no higher ranks as it was unclear how these should be grouped with no representatives of many orders and families sequenced. As data on a wider range of orders, families and genera have become available, a system of two subphyla, 15 classes, eight subclasses, and 60 orders has been developed and is now commended for general use [24]. Perhaps the most unexpected result to traditional mycologists was that the concept of there being two major classes of ascomycetes, ascohymenial and ascolocular, has had to be rejected [49]. Further, a full integration of lichenized groups, something generally not attempted by mycologists before the mid-1980s, was critical to an understanding of relationships in these fungi, though many mycologists only reluctantly started to admit this and consider these fungi. It came as a shock to many when it was reported that major fungal lineages in ascomycete fungi were derived from lichen-forming ancestors [52]. In the light of yet further data, it has become clear that some lichen groups occupy a basal position amongst all those ascomycetes that form fruit bodies, and it has been suggested that the first such ascomycetes were lichenized [25]. Ascomycetes not forming fruit-bodies appear to be even more ancient, especially some groups of yeasts (*Saccharomycetales* and *Schizosacharomyces*) and the much misinterpreted *Pneumocystis*, sometimes collectively referred to as *Archiascomycetes*, but as yet of somewhat uncertain position [24].

Of course with molecular phylogenetic approaches it is possible to place a fungus which is only known to produce asexual conidia into the overall fungal system in the absence of any asci, let alone fruit bodies. Consequently, units such as *Coelomycetes*, *Deuteromycetes*, *Fungi Imperfecti*, and *Hyphomycetes* are falling into disuse as they do not represent monophyletic units. These fungi are now being increasingly covered within the pertinent sexual groups in texts [e.g. 44,53], and referred to informally as "anamorphic", "conidial", or "mitosporic" fungi. In the current edition of *Ainsworth & Bisby's Dictionary of the Fungi* [45], there are consequently entries such as *Cladosporium* Link (1816), anamorphic *Mycosphaerella* and *Dactylosporium* Harz (1872), anamorphic *Ascomycetes*.

Molecular data have also enabled some completely sterile genera, not even forming conidia, to be placed in the overall system of classification. For instance the positions of some sterile lichen-forming genera have now been resolved, including the placement of *Coscinocladium* in *Physciaceae* [15] and *Lepraria* (most species) in *Stereocaulaceae* [22].

In order to accelerate the move from turmoil to a position of increased stability in overall fungal classification, a major collaborative international initiative involving over 120 participants from more than 20 countries was embarked upon in 2003, Assembling the Fungal Tree of Life (AFTOL). This bold project aims to sequence nine genes in about 1,500 species, carefully selected so as to cover all fungal groups [51,71].

¹ Referred to as "Stramenopiles" or "Stramenipiles" but validly published as "*Straminipila*" [18].

² Sometimes also referred to as the kingdom *Mycota*, but *Fungi* is the more appropriate kingdom name as the suffix "-mycota" indicates the rank of phylum. In this contribution all formal scientific names are placed in *italic* type, as is the practice in the International Code of Botanical Nomenclature [30] which also regulates the nomenclature of fungi.

³ 15 September 2005.

All change in genera

Turmoil in the hierarchical classification is perhaps somewhat academic and remote to most people working with and recording fungi. This is not so at the generic level when changed views on classification can necessitate the adoption of new and unfamiliar names. As names are the key to access all that is known of a fungus in the literature, changes inevitably hinder communication and data retrieval. Despite the irritation such changes cause, if they are soundly based and mean that relationships are being clarified, they should be welcomed as the resultant genera will be more natural (monophyletic) and so of greater value in predicting the properties of the constituent species. Three examples may serve to illustrate different situations that can be uncovered.

In the mushroom genus *Coprinus*, the species were found to belong to four genera in two different families: *Coprinus* (three species) in *Agaricaceae*; and *Coprinopsis* (100), *Coprinellus* (42) and *Parasola* (18) in *Psathyrellaceae* [62]. The lichen-forming ascomycete genus *Parmelia* was divided into 38 genera through the 1970s into the mid-1990s, mainly based on vegetative and secondary chemical characters; molecular studies to date suggest the recognition of 16, which also correlate with fundamental features such as cell-wall carbohydrates, ascospore and conidium types, ultrastructural features of the cortex, and ecological requirements [3-5]. In one section of the conidial genus *Verticillium*, five genera have been found to justify segregation as a result of molecular phylogenetic studies; most are strongly correlated with the hosts they attack, for example, rusts, rotifers, nematodes, insects and other fungi, and plants [79].

Another shock has been the proof by molecular studies that three species of the discomycete genus *Stictis* on *Populus* in Scandinavia live as saprobes on wood, but if they establish on bark they form crustose lichens traditionally placed in a separate genus, *Conotrema* [76]. As there are at least 53 genera⁴ which include either lichen-forming and lichen-dwelling (lichenicolous) species and also ones on other substrates, this should not have been too much of a surprise, but to have at least one case proven molecularly suggests that similar cases may also be soundly based.

The issue of assigning separate names to different stages in the life-cycle of a single fungus species has been controversial since the 1930s. It was recognized early in the molecular era that a *Penicillium* with no known sexual stage could be assigned with confidence, for example, to *Talaromyces* on the basis of sequence data, so why maintain two names [50]? This suggestion met with little support at the time, but now proposals have been made not only to limit future use of dual nomenclature [34], but to abandon it altogether [64]. The issue is now being examined by a Special Committee on Pleomorphic Fungi established by the International Botanical Congress in Vienna in July 2005; it is mandated to report in 2011.

A proliferation of cryptic species

During the 1980s into the mid-1990s, considerable attention was directed to the recognition of incompatibility groups within morphologically defined species, from mushrooms [e.g. 61] to pathogenic moulds exemplified by *Fusarium* [e.g. 14] and *Rhizoctonia* [e.g. 70], i.e. popula-

tions which were reproductively isolated and behaving as biological species. How such cases should be handled occasioned some controversy [7,61]. There was merit in distinguishing such populations where they differed in distribution, host range, or pathogenicity. The advent of molecular phylogenetics, however, has provided an independent test as to whether there were substantial genetic differences between such incompatible populations, and a means of assessing the genetic homogeneity and validity of a species concept even when they could not be grown in pure culture.

In a large proportion of cases where substantial numbers of strains of the same morphologically circumscribed species are examined, they are found to contain more than one phylogenetic species. When many of the clades which are revealed as previously hidden "cryptic" species are re-examined morphologically, it is common for characters which had been overlooked and (or) regarded as part of the within-species variation to be found to correlate with these newly recognized species. Examples are almost boundless, and occur across a wide range of fungal groups.

Amongst macromycete basidiomycetes, the honey fungus *Armillaria mellea* s. lat. provides the classic case (Figure 1A); the number of recognized species in Europe rose as a result of such studies from one to eight [60], and additional species continue to come to light, for instance two more recently from Africa [13]. In the chanterelle genus *Cantharellus*, one of the sought after edible species in North America proved distinct from the European *Cantharellus cibarius*, but also is more brightly pigmented [21]. The taxonomic situation over the medicinally important *Ganoderma lucidum* has yet to be formally resolved, but it is evident that several distinct species are involved [40].

In the lichen-forming genus *Parmelia*, three cryptic species have now been discovered within *Parmelia saxatilis* in Europe (Figure 1C), and are also distinguishable by morphological and ecological characteristics [55]. Three genotypes of the closely allied *Parmelia sulcata* were recognized in the UK [16], and a population differing in rhizine type has since been described as a separate species from central Europe [19]. In the related genus *Punctelia*, one long-synonymized species has been resurrected, and three yet to be formally named discovered [17].

The recognition of three species and two subspecies in the Dutch elm disease fungus *Ophiostoma ulmi* s. lat. that has caused such devastation of elm tree populations in Europe and North America, continues to be confirmed by additional sets of molecular data [29,59]. While papers in one recent issue of *Mycological Research* showed that the "single species" *Ceratocystis polonica* [54] and *Leptographium lundbergii* [41] each contained three species.

In *Fusarium*, which has a long history in the recognition of incompatibility groups and "special forms" able to attack particular plants, numerous cryptic species are being confirmed. For instance, nine species have now been recognized within *Fusarium graminearum*, almost all of which are also separable by cultural characteristics or the details of their conidia [58]. Similarly in *Trichoderma* (Figure 1D), the number of accepted species increased from nine in 1969 to 33 by 1998 [27], and is currently rising almost exponentially; for example, 20 more species were added in a single publication in 2003 [12]. In that genus, not only are most of the species distinguishable by

⁴ Hawksworth [36] lists 48 genera, to which should be added *Aulaxina*, *Chroodiscus*, *Epibryon*, *Gyalideopsis*, and *Muellerella*; *Paranectria* was a lapsus for *Trichonectria* in that list.

morphological and cultural features, many also have different sexual stages. There has been much confusion in the past over even economically important species in *Trichoderma*. Strains used in biocontrol generally named as *Trichoderma harzianum* belong to at least four species [48], and even that causing a severe problem in mushroom beds has only recently been recognized as distinct [66].

In the pneumonia-causing *Pneumocystis carinii*, the originally single species was suggested to comprise at least eight, not then formally named, by Eriksson [23]. It now appears that species in the genus are specific to different mammalian hosts [72], with that infecting humans now described as *Pneumocystis jiroveci* [73], and one recently recognized in laboratory mice [43]. Could this one species really represent 4,260, the number of known mammals?

Especially fascinating has been the power of molecular techniques to conclusively identify cases of hybridisation between different species of fungi. Perhaps the classic instance is the poplar rust *Melampsora* $\times$ *columbiana* in western North America which arose when populations of *Melampsora medusae* and *Melampsora occidentalis* came into contact to produce a particularly pathogenic hybrid [57]. Parallel situations appear to have taken place in bunt fungi of wheat, *Tilletia caries* and *Tilletia foetida* [6], and between the Dutch elm disease fungi *Ophiostoma novo-ulmi* and *Ophiostoma ulmi* (Figure 1B) [8].

Since evidence was produced from several independent data sets to suggest that there might be 1.5 million species of fungi on Earth, of which only 5-6% were yet named [31], there has been the issue of "Where are the missing fungi?" It is now becoming abundantly clear that many of the predicted missing species are hidden under single species names [32,37].

Discussion

Traditional systematists unprepared to undertake or integrate molecular data into their studies see the move to the use of such information as a threat, while those that embrace it find it stimulating and enlightening. It is not so much an issue of "Molecules vs. Morphology", but "Molecules + Morphology". Yet to achieve the "+" almost always involves collaboration; taxonomists that really know the minutiae about the morphology, ecology, and distribution of the fungi themselves, working together with specialists versed in the cutting edge methodologies of molecular phylogenetic approaches and the interpretation of the results. This is a very different way of operating to the widespread image of the taxonomist sitting alone in a dusty room in some museum, peering down a microscope or clutching a large hand lens, and surrounded by boxes of material and mounds of books and papers. But co-operation is becoming the norm. Of the 1,368 new scientific names for fungi listed in the July 2005 issue of the *Index of Fungi*, 786 (57.5%) were introduced by more than a single author. In contrast, of the 1,177 names in the July 1985 issue, only 458 (39%) were co-authored.

But the contribution of molecular methods to systematic biology is not only a matter of resolving evolutionary relationships and preparing revised classifications in particular genera or species. It is causing a currently ongoing fundamental reassessment of the systematic importance of characters such as fruit body types and developmental patterns, and at the same time a renewed interest in ultrastructure, micromorphology, cultural, physiological, pathological, and ecological features as these increasingly correlate with the phylogenetic output. Molecular methods have provided the tools necessary to test long-cherished

hypotheses objectively in the best scientific tradition. In consequence taxonomy is becoming less of an art and more of a science.

A significant other pleasure to emerge from Pandora's box is the provision of tools to facilitate species recognition, even when the fungi are not sporing and uncultured. This will become increasingly significant in identification as the number of species that have sequences deposited in GenBank swells from the current 13% (p. 128), and especially with the targeted sequencing of the AFTOL project (p. 128). However, there is no room for complacency. The number of new fungi described each year (*ca* 1,100) exceeds the annual additions to GenBank, consequently it will require a considerable investment to increase the percentage of species represented. Another new initiative may also facilitate molecular identification

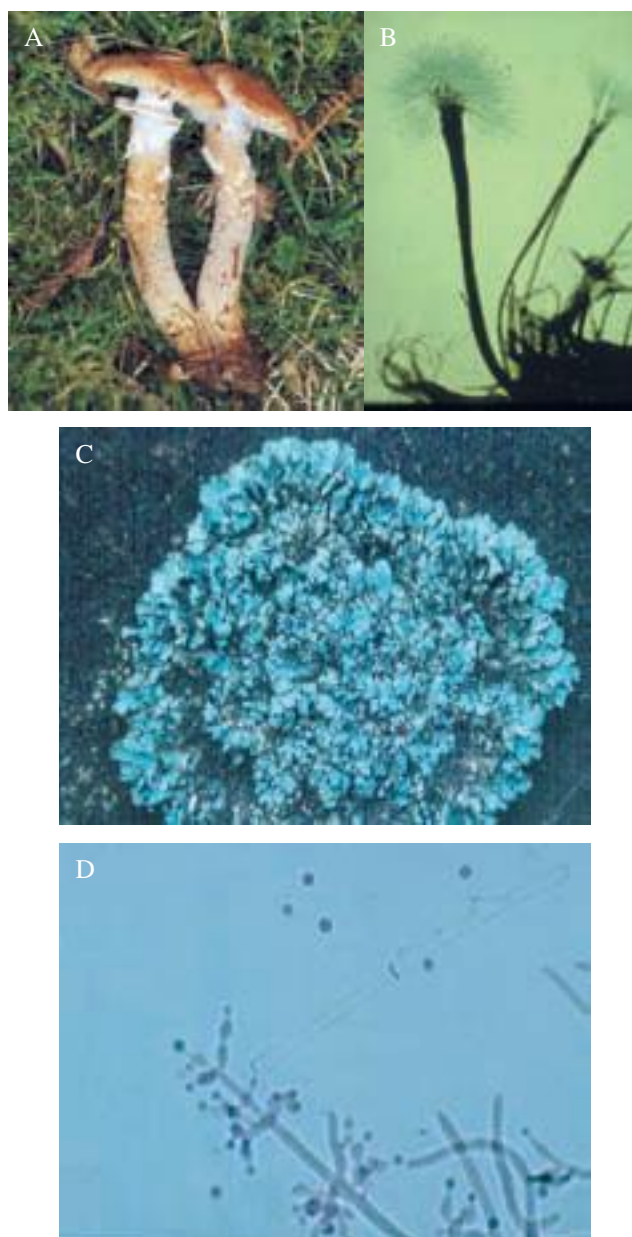


Figure 1. Examples of "species" of fungi which have been found to comprise several cryptic species: (A) *Armillaria mellea* s. lat. (*A. ostoyae* illustrated); (B) *Ophiostoma ulmi* s. lat. (*O. novo-ulmi* anamorph stage figured); (C) *Parmelia saxatilis* s. lat. (*P. saxatilis* s. str. figured); and (D) *Trichoderma harzianum* s. lat. (*T. aggressivum* figured).

Table 1. Species from which genomes are expected to be sequenced by the end of 2006.

<i>Ashbya gossypii</i>	<i>Hansenula polymorpha</i>	<i>Phytophthora sojae</i>
<i>Aspergillus clavatus</i>	<i>Histoplasma capsulatum</i>	<i>Piromyces</i> sp.
<i>Aspergillus fennelliae</i>	<i>Kluyveromyces lactis</i>	<i>Pneumocystis jiroveci</i>
<i>Aspergillus fischeri</i>	<i>Magnaporthe grisea</i>	<i>Rhizopus arrhizus</i>
<i>Aspergillus flavus</i>	<i>Melampsora larici-populina</i>	<i>Saccharomyces cerevisiae</i>
<i>Aspergillus fumigatus</i>	<i>Mycosphaerella fijensis</i>	<i>Schizophyllum commune</i>
<i>Aspergillus nidulans</i>	<i>Neurospora crassa</i>	<i>Schizosaccharomyces pombe</i>
<i>Candida albicans</i>	<i>Paracoccidioides brasiliensis</i>	<i>Sporobolomyces roseus</i>
<i>Candida dubliniensis</i>	<i>Phanerochaete chrysosporium</i>	<i>Trichoderma reesei</i>
<i>Candida glabrata</i>	<i>Phycomyces blakesleeanus</i>	<i>Trichoderma virens</i>
<i>Coccidioides immitis</i>	<i>Phytophthora capsici</i>	<i>Ustilago maydis</i>
<i>Coprinopsis cinereus</i>	<i>Phytophthora infestans</i>	<i>Xanthoria parietina</i>
<i>Cryptococcus neoformans</i>	<i>Phytophthora ramorum</i>	<i>Yarrowia lipolytica</i>
<i>Debaryomyces hansenii</i>		

if taken up by mycologists generally. The Consortium for the Barcode of Life (CBOL; www.barcoding.si.edu) established in 2004 aims to sequence short portions of a standardized region of the genome for all forms of life to facilitate rapid low-cost identification by non-specialists [69]. The first fungal genus in which the barcode approach has been applied is *Trichoderma* (teleomorph *Hypocrea*), which enables 75 species to be identified [20].

However, the value of sequence data is totally dependent on accurate identification of the organism used. This is a major problem in public databases such as GenBank where it has been estimated that up to 20% of the fungal deposits may be wrongly named [9]. One response to this difficulty has been the establishment of separate databases managed by specialist groups and where voucher material is preserved of the fungus sequenced. These already exist for *Fusarium* (FUSARIUM ID [28] and *Trichoderma* (TrichoBLAST [47]), and one is being developed for ectomycorrhizal fungi (UNITE [46]). The intimate involvement of specialist taxonomists with molecular mycologists is essential in the development of all reliable sequence-based systems. In any case it is critical that voucher material is deposited in publicly accessible genetic resource collections or other reference collections so that identities can be verified [1,35].

Especially exciting in the future will be the ability to compare whole genomes. By the end of 2006, 40 fungi will have had their complete genomes sequenced (Table 1), and it is anticipated that additional species will be added to the US Department of Energy's Joint Genome Institute schedule for 2007 (www.jgi.doe.gov/sequencing/). At present the process is still relatively time-consuming and expensive, but the technology has accelerated dramatically. Within the next 10–15 years, however, I am confident that more surprises will emerge from the box as whole genomes are compared. The genes responsible for different characters will be identified, and it will be possible to determine if some organisms contain particular genes but they are generally or always switched-off. Such comparisons are likely to provide even more exciting insights into characters used in classification, such as abilities to use certain substrates, produce distinct extrolites⁵, or form particular morphological structures from types of spore ornamentation to fruit body types.

Molecular approaches are also contributing to our knowledge of unexplored fungal diversity. Examples are studies on soils where novel clades at the subphylum or class level have been reported from tundra soils in Colorado [67]; isolates from rock surfaces in Mallorca [65] and the Antarctic [68]; and insect hind-guts which yielded 200 undescribed yeasts, equivalent to 30% of all known yeasts, from just two sites [75]. Some of the “unexplored habitats” identified as one of the sources of the “missing fungi” [37] are being revealed through molecular approaches.

The issue is consequently not so much one of molecules *versus* morphology, but the need for a wholesome marriage in which the partners fully respect and input their complementary skills in a synergism, taking the subject to new heights. The Pandora's box of molecular surprises is to be seen as one of blessings and not one of miseries and evils, though reaping those blessings can involve short-term pain.

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⁵ The term preferred by J.C. Frisvad for extracellular produced compounds including “secondary metabolites”, and first used in print in Mycol. Res. 2004; 108: 597.

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