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New views on fungal evolution based on DNA markers and the fossil record

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Abstract

Molecular markers have facilitated a better understanding of the evolution of fungi. Molecular phylogenetics determined the closest relatives of fungi and defined natural groups within the true fungi. The impact of molecular markers on the population biology of fungi has been enormous, helping to define cryptic species and elucidating fungal breeding biology. The interaction between molecular phylogenetics and the fungal fossil record is discussed.

Keywords: Fungi; Evolution; Molecular phylogeny; Population biology

1. Introduction

Fungi have traditionally been defined as a group of eukaryotic, achlorophyllous organisms with adsorptive nutrition and filamentous somatic structures, known as "hyphae" or "mycelium" [1]. It has become clear that several phylogenetically unrelated eukaryotic lineages fall within this disparate array of organisms traditionally studied by mycologists. One lineage is known as the true fungi [7], comprising the fungi forming macroscopic fruiting structures and many others. This group is monophyletic, i.e., it contains all the descendants of one common ancestor.

Fungi are found in all ecosystems and show a great diversity of lifestyles. It has been estimated there may be as many as 1.5 million species of true fungi, but only 69 000 have been described so far [13]. They live as saprobes, decaying dead organic material, or in symbiosis with other organisms. Symbiotic interactions range from parasitism, as exemplified by the huge variety of fungal diseases, to mutualistic interactions, benefiting both partners. Plants are the symbiotic partners in the mycorrhizal interactions. Green algae and cyanobacteria participate in the lichen symbiosis. Insects form many different kinds of fungal symbioses. Examples are the fungus gardens of attine ants, Trichomycetes lining the inside of insect guts or the symbiosis between fungi and Ambrosia beetles, which have special pouches to carry their fungal symbionts [1]. Even ruminants are among the symbiotic partners of fungi, harboring chytrid fungi in their digestive tract.

Morphological characteristics at the macroscopic and the light microscopic level that can be used for classification are often scarce in fungi. Therefore, views of their evolutionary relationships have been obscured by convergence and homoplasy in many cases. With the advent of electron microscopy, a wealth of more useful characters became available, but in many cases these were still problematic to interpret phylogenetically. In the past few years, phylogenetic analyses of DNA sequences have offered the opportunity to elucidate fungal phylogeny without the need to interpret phenotypic characters and provided numerous new insights into fungal evolution. Recent fossil findings of fungi allowed a better understanding of the evolutionary history of fungi. Fossil record and molecular data continue to complement and challenge each other.

2. What is a fungus?

With the rise of molecular phylogenetics, fungi *sensu stricto* (the true fungi) were defined as a monophyletic group [7]. This group comprised Basidiomycota, Ascomycota, Zygomycota and Chytridiomycota.

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Based on biochemical and ultrastructural characters it had already been clear that several groups traditionally treated as fungi *sensu lato* are not a part of the true fungi [1]. DNA sequence data have made it possible to place these groups in other major eukaryotic clades defined by molecular phylogenetics, where this was not already possible by morphological analyses.

The Oomycota, for instance, are members of the Stramenopila clade, although they resemble the true fungi closely in their growth form, their adsorptive and parasitic lifestyles and the formation of spores. The Stramenopila are a major eukaryotic lineage comprising brown algae, diatoms and chrysophytes [26]. Some members of the Oomycota are known as "water molds", some are prominent pathogens, e.g., *Phytophthora* or *Peronospora*. The Oomycetes are a fascinating example of convergence to the true fungi. These two groups of organisms adapted a similar lifestyle although they are not related at all and evolved independently. Interestingly, no mutualistic symbioses resembling mycorrhizae and lichens are known from this group. It will be interesting in the future to compare the cell biology of true fungi and Oomycota to find the reason for this apparent difference.

There is now compelling evidence that the closest known relatives of the true fungi are animals [2], and not green plants, in spite of the historically-grounded responsibility of botanists for fungi. The sister group relationship of animals and fungi is supported by phylogenetic analysis of several independent genes and by a characteristic insertion in the elongation factor alpha gene [2]. It can be speculated that both groups arose from one-celled heterotrophic organisms similar to another group that forms a monophyletic clade with animals and fungi, the choanoflagellates. These resemble both the earliest branch of animals, the sponges, and the earliest branch of the true fungi, the chytrids [3].

Another recently defined group of organisms branching close to the animal-fungal divergence are the Ichthyosporea, an enigmatic group comprising parasites of marine animals. Initially, they were informally named "DRIPs" after the first known members of this group (*Dermocystidium*, the "Rosette agent", *Ichthyophonus*, and *Psorospermium*) [27]. Since that first study, a number of additional members of the group have been discovered, and the group was named Ichthyosporea [9]. Some of these parasites have fungus-like thalli and sporangia. That is also the case for *Amoebidium parasiticum*, which was most recently reported as a member of the Ichthyosporea [4]. *Amoebidium* was previously thought to be a Trichomycete (Zygomycota), based on its habitat as arthropod symbiont and the thallus morphology.

Hardly any problem case of molecular phylogenetics can be imagined to be as controversial as the Microsporidia. This enigmatic group of unicellular intracellular parasites was thought to be one of the deepest-branching eukaryotic lineages from rDNA analyses [39], and the absence of mitochondria seemed to suggest that Microsporidia are a primitive group of Eukaryotes which may have arisen before the establishment of the mitochondrial endosymbiosis. However, most protein genes, among them tubulins, placed the Microsporidia within the fungi [20], although it was not possible to determine their closest relatives. These findings indicate that an extremely increased mutation rate in the ribosomal genes caused the artifact of phylogenetic analysis known as long branch attraction, resulting in a basal placement of this group within the eukaryotes. The discordant results highlight possible problems that can arise when studying major eukaryotic lineages with single-gene phylogenies. Ribosomal DNA is still the gold standard of phylogenetic analysis and will continue to be so, but to elucidate the relationships among major eukaryotic clades it is necessary to employ alternative phylogenetic markers (e.g., elongation factor alpha, tubulins, actin).

3. Many fungal lifestyles and morphologies arose several times (and were lost again)

Molecular phylogenetics showed that phylogenetic relationships of the "lower fungi" (Zygomycota/Chytridiomycota) are generally more complicated than previously thought. Zygomycota have a non-septate mycelium and during sexual reproduction they form typical structures known as zygospores. The Chytridiomycota are characterized by motile reproductive cells that have a single, posteriorly directed flagellum. They are thought to be the most ancient fungal group, because they retained this and other features characteristic of life in the water [1]. Occasionally, the Chytridiomycota were placed in the protists but molecular phylogenetics confirmed that they are true fungi [7].

However, there is some evidence that both Zygomycota and Chytridiomycota are not monophyletic [18]. Both comprise several diverse lineages which are only distantly related. Molecular phylogenetics of the Zygomycota and Chytridiomycota has not been without problems because of the ancient age of the clades and the drastically increased mutation rates in the ribosomal RNA of some Zygomycota lineages. These lineages cause very long branches in phylogenetic trees and their placement tends to be problematic. Therefore it may be misleading to rely on rRNA alone to elucidate the relationship of the deep branches within the true fungi. A comprehensive analysis of Zygomycota and Chytridiomycota, using multiple genes, remains to be done, but studies of subgroups of these phyla yielded interesting results.

In the Zygomycota, the Mucorales, a group of ubiquitous saprophytes, have been studied using a combination of sequence data from small and large subunit rDNA and elongation factor alpha [25]. The striking result was that most of the taxa on the family level that were defined by classical morphological criteria were not monophyletic. Even the often-studied genera *Mucor* and *Absidia* were polyphyletic. This was interpreted as widespread convergence of the asexual reproductive characters used to establish previous classifica-

tion schemes. The evolutionary relationships among major subgroups of Zygomycetes (Mucorales, Entomophthorales, Trichomycetes and others) are currently not well resolved.

As judged from rDNA sequences, there appears to be a monophyletic core clade in the Chytridiomycota which is the most ancestral group in the true fungi, but it is not well supported by bootstrap analyses [18]. At least one subgroup of the Chytridiomycota in the traditional sense, the Blastocladiales, groups in the Zygomycota. This order comprises *Allomyces macrogynus*, a popular experimental organism which has been used to study fungal pheromones [1]. Phylogenetic analyses using different genes are in good agreement indicating that the Blastocladiales are a zoosporic lineage that evolved independently from the rest of the chytrids. It has therefore become clear that flagella have been lost several times independently during fungal evolution [5]. As independence from water is a major adaptation to life on land, this is in fact not surprising.

The data are less conclusive for *Basidiobolus ranarum*, which has been traditionally placed in the order Entomophthorales of the Zygomycota based on its morphology. This is supported by its beta tubulin sequence [20]. However, according to rDNA sequences, it appears to a be a member of the monophyletic chytrid core clade [18].

Ascomycota and Basidiomycota are the two groups of true fungi which are also sometimes called "higher fungi", because some of them form macroscopic fruitbodies (mushrooms). Together, they are in a monophyletic clade that was termed "Dikaryomycotina", which is united by a dikaryotic life stage and regular septation of the hyphae [1] and supported by rDNA phylogenies [5]. According to most rDNA phylogenies, arbuscular mycorrhizal fungi (Glomales) are the sister group to the dikaryomycotina [5,11]. The Glomales were traditionally placed in the Zygomycota. The absence of zygospores and the growing evidence that the Glomales are fundamentally different from all other Zygomycetes was the reason to erect a separate phylum Glomeromycota [32]. Interestingly, the fungi in this Asco-/Basidio-/Glomeromycota clade are very often associated with plants. With only one known exception (the ectomycorrhiza of the Zygomycete *Endogone*), plant-fungal mutualisms are only formed by this group.

It is well-established that the yeast growth form was adapted by numerous unrelated groups of Asco- and Basidiomycota [34]. Many dimorphic fungi live as a yeast under certain conditions or in certain life stages and also have a filamentous form. However, even much more complex structures formed by fungi evolved multiple times independently.

Hibbett et al. [17] performed a comprehensive phylogenetic analysis of a subgroup of Basidiomycota, many of which are mushroom-forming, to elucidate the evolution of fruitbodies. These authors showed that mushrooms with gilled caps evolved at least six times independently from morphologically diverse ancestors. Fruitbodies enclosing their spore-bearing structures ("puffballs" and alike) evolved at least four times. Bruns et al. [6] had already shown earlier that some false truffles evolved very rapidly from mushroom-like ancestors. However, there is no evidence so far that puffballs and other gasteromycetes evolved back into open fruitbodies. This easily adopted growth form apparently is an evolutionary dead end.

Another example of how misleading fungal morphology can be was reported in arbuscular mycorrhizal fungi (Glomeromycota). One fungus was reported to produce two types of spores that were each characteristic of a different genus and family. The two spore morphs were known as *Acaulospora gerdemannii* and *Glomus leptotichum*. Using rDNA sequences it was shown that the fungus did not belong to any of the two genera *Glomus* and *Acaulospora*, but instead belonged to a lineage that is deeply divergent within the Glomeromycota and ancestral to the previously known groups [29].

Fungi adapt new lifestyles with equal ease as they change morphologies. One of the most fascinating lifestyles of fungi are lichens. These dual organisms are composed of an alga or a cyanobacterium and a fungus, but the fungus mostly determines the morphology and, therefore, the classification. Using molecular phylogenetics, Gargas et al. [10] concluded that at least five groups of fungi in Ascomycota and Basidiomycota adopted this lifestyle independently. A more recent study showed that during the evolution of the Ascomycota relatively infrequent gains of lichenization have been followed by multiple losses of that lifestyle. Therefore, major Ascomycota lineages without any lichen-forming members are derived from lichenized ancestors [22].

In the symbiosis between plants and fungi which is known as mycorrhiza, fungi act as an extended root system for the plant and receive carbohydrates in exchange. The fungal symbionts of arbuscular mycorrhiza (AM), the oldest of these associations, were already present when the first simple plants colonized terrestrial ecosystems in the Ordovician [28], and there is direct evidence from the Devonian for the presence of this symbiosis [31]. Consequently, this symbiosis is present in almost all major lineages of land plants and represents the ancestral type of mycorrhiza. The only member of the Glomeromycota which is currently assumed to be non-mycorrhizal is *Geosiphon pyrifome*. This enigmatic fungus forms a peculiar symbiosis with cyanobacteria, harboring *Nostoc punctiforme* in bladders produced on the surface of soils [11]. Under the assumption that arbuscular mycorrhiza was not adopted independently by four different clades of Glomeromycota, molecular phylogenetic data [32] suggest that this "zygomycete lichen" is probably derived from mycorrhizal ancestors and not their precursor.

Considerably later during the evolution of land plants, other mycorrhiza types arose with members of Basidiomycota and Ascomycota as mycosymbionts. For example, many woody plants form ectomycorrhiza, the dominant plant-fungal symbiosis in temperate and boreal forests. Interestingly, the ectomycorrhizal lifestyle was adapted multiple times in the Ascomycetes and Basidiomycetes by unrelated

groups [15]. In the same study, it was also demonstrated that the symbiosis was abandoned again in several clades in favor of saprophytism.

4. Fossil history

Although considerable progress has been made recently in uncovering the fossil record of fungi, the findings are relatively scarce when compared to other groups of organisms. The actual vegetative body, the mycelium, is usually not well preserved and several other groups of organisms produce structures that can be mistaken for fungal mycelium.

The oldest macroscopic fungus in the popular sense, a "mushroom", was found embedded in amber from the Cretaceous (90–94 Myr). This fruitbody shows all the characteristics of today's Basidiomycete genera *Marasmius* or *Marasmiellus* [16]. Molecular evidence suggests that the first mushrooms may have evolved much earlier [5] and older specimens can be expected to be found in the future.

Many of the oldest and most well-preserved fungal fossils were found in association with plant fossils in the early Devonian Rhynie Chert (400 Myr). Within some of the plants from this site, arbuscules were found [31]. These small, tree-like structures are formed by arbuscular mycorrhizal fungi within their plant hosts. Therefore, these fossils are the oldest direct evidence of the arbuscular mycorrhizal symbiosis.

The Rhynie Chert also contained a wealth of specimens from other fungal groups. Among them are a blastocladalean chytrid, strikingly well-preserved and very similar to today's genus *Allomyces* [38]. Another fungus has fruiting structures resembling today's Ascomycete groups of Pyrenomycetes or Loculoascomycetes. The quality of the specimens even led to distinguishing individual asci, the microscopic sac-like structures containing meiospores [37].

A possibly older Ascomycete was found in the the Silurian of Sweden [33]. The specimens resemble extant Ascomycetes found in arthropod frass. This finding is compatible with recent rDNA molecular clocks [5,28]. Together with the Devonian Ascomycetes from about the same time, it indicates that the radiation of Euascomycetes had already started at that time.

The oldest fungi known at the present time are glomalean fungi from the Ordovician [28]. These specimens were found in dolomite rock from Wisconsin, which is between 460 and 455 million years old. At that time, the land flora only consisted of plants on the bryophytic level. Thus it is intriguing to speculate that the early glomalean fungi formed associations with bryophytes and may have played a role in the colonization of land. These fossils are also useful for calibrating molecular estimates of the time course of fungal evolution. Using the glomalean fungi from the Ordovician and a number of other fungal fossils as calibration points for an rDNA molecular clock, it was estimated that Asco-/Basidio- and Glomeromycota diverged from each other about 600 to 620 million years ago, which is in good agreement with previous estimates [5]. Together, the fossil record and these molecular data provide a fairly consistent picture of fungal evolution. Molecular phylogenies have also demonstrated that there are older lineages within the true fungi than the Glomeromycota and their fossils remain to be found in the future.

Another recent molecular clock study, using a large number of different genes, suggests that fungi and land plants are substantially older than previously thought [14]. The split of Basidiomycota and Ascomycota was estimated at 1208 Myr, the origin of Glomeromycota between 1200 to 1400 Myr, and the divergence of mosses from vascular plants at 703 Myr. This molecular clock was calibrated using another molecular estimate of animal evolution based on the divergence of birds and mammals (310 Myr). These results are difficult to accommodate with the fossil record of plants and fungi and it remains to be seen whether they can be substantiated.

5. Are morphologically asexual fungi having sex?

Many fungi are only known in their asexual stage. In the days before electron microscopy and molecular biology they could only be placed in the artificial group of Deuteromycetes (Fungi Imperfecti) and classified by the morphology of the asexual stages. This group has been vanishing, because molecular sequencing data made it relatively easy to find the closest sexual relatives of asexual forms.

For many fungi, however, it is still not known whether they are truly asexual or whether they simply do not happen to form the sexual stage when observed. Molecular biology now enables us to examine the population structure and the reproductive mode of putative asexual fungi by multilocus genotyping. Within a fungal population, sequence markers from several independent genes are analyzed phylogenetically under the parsimony criterion. In the presence of recombination, the different genes have different evolutionary histories and their phylogenies conflict, because of the horizontal transfer of genetic information. This lack of association is detected by the phylogenetic methods or alternatively quantified by the index of association coefficient [35].

Surprisingly, using this method, a recombinant population structure was detected in a number of fungi that have never been reported to form a sexual stage. Among them are *Coccioides immitis*, the cause of California "valley fever" [8], and *Aspergillus flavus*, a fungus producing highly carcinogenic toxins on foods, for instance on peanuts [12]. These results show that even fungi without a sexual stage may in fact follow mixed strategies with components of recombination and clonality.

It is unknown where, how and how often recombination occurs in these fungi in the absence of sexual structures. However, it has to be taken into account that, unlike the sit-

uation in many other organisms (e.g., plants or animals), the vegetative cells of fungi may fuse with each other, forming a heterokaryon. In these heterokarya, during a process known as parasexuality, nuclear fusion, mitotic crossing-over and haploidization can occur. However, it is assumed that parasexuality is rare in natural populations [1] and it is unclear whether it could account for the recombinant population structure in fungi lacking sexual structures.

6. The hidden diversity: cryptic species

As mentioned before, it has been problematic in many cases to determine the phylogenetic relationships of fungi by morphological methods. This is of course also true with recognizing the fundamental unit of the Linnean system, the species. Morphological species recognition is strongly biased by human perception and severely limited in organisms with scarce morphological characters to discern. Under a biological species concept, on the other hand, a species is a group of populations which is reproductively isolated from other groups. Traditionally, biological species were determined in fungi by mating tests. This is highly problematic, because many fungi cannot be cultivated readily, and, as outlined in the previous section, many more do not mate in the laboratory. Molecular methods now enable circumventing this problem by detecting gene flow and reproductive isolation.

Using phylogenetic methods, evidence for hidden fungal diversity near the species level has been found in a number of cases, even with well-studied organisms. O'Donnell et al. [23] reported that *Fusarium oxysporum* f. sp. *cubense*, causing the economically important Panama disease of bananas, is in fact a polyphyletic assemblage of five different lineages of fungi.

Fingerprinting techniques using anonymous markers, e.g., amplified fragment length polymorphisms, can also be used to differentiate species. Redecker et al. [30] presented a statistical approach to determine whether fungal fruitbodies were formed by the same clone (genet) and belonged to a randomly interbreeding population. Contrary to most other studies mentioned here, in this study the molecular markers united two putatively separate fungal morphospecies of *Russula* occurring in the same site by showing that they were genetically not differentiated.

Diagnosing species borders from single-gene phylogenies can be difficult because usually the markers do not indicate a clear cutoff between species and higher taxonomic groupings. Therefore an approach termed genealogical concordance phylogenetic species recognition (GCPSR) was introduced [36]. It is based on similar methods using multilocus genotypes as described above for detecting recombination. Incongruity of gene genealogies within a given group indicates gene flow, and delimits a species. As the approach detects reproductive isolation, the resulting groups also fulfill the criteria of the biological species concept. One disadvantage of the method is that it has difficulties with truly clonal species, but as described above, many fungal populations appear to show at least some component of recombination. Similar to mating tests, GCPSR in almost all cases detected more than one biological or phylogenetic species within phenotypically-defined species, indicating that the total number of fungal species is currently underestimated by at least a factor of two. For example, two reproductively isolated groups were detected within *A. flavus* with the gene genealogy approach. One of the groups showed clear evidence of recombination [12]. *Aspergillus oryzae*, a closely related fungus that is used industrially, e.g., to ferment soy sauce, was reported to be nested among the clonal lineages. Within *Histoplasma capsulatum*, a pathogen of humans and animals, six cryptic species were found [19]. *C. immitis* contains two reproductively isolated groups [21]. O'Donnell et al. [24] reported seven cryptic species within *Fusarium graminearum*, the agent causing wheat scab. Findings like these will be important in the future for developing strategies to control these pathogens.

7. Future perspectives

Molecular markers now allow the study of phylogeny and population biology of fungi in greater detail than ever before. Applying the new techniques will provide a better understanding of the processes of speciation and adaptation in this group. Based on more refined species definitions, the number of fungal species can be expected to at least double and the true biodiversity of fungi will become more obvious. A more natural classification of fungi also will enable building better molecular tools for detecting and identifying them and thus elucidating their role in ecosystems. Palaeobiology has been in an interesting exchange with molecular phylogenetics and will hopefully continue to do so in the future. As indicated by molecular data, the oldest fungal fossils found so far are not from the earliest fungal lineages and older fungal fossils may yet remain to be found.

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