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Fungal Diversity Revisited: 2.2 to 3.8 Million Species

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ABSTRACT The question of how many species of *Fungi* there are has occasioned much speculation, with figures mostly posited from around half a million to 10 million, and in one extreme case even a sizable portion of the spectacular number of 1 trillion. Here we examine new evidence from various sources to derive an updated estimate of global fungal diversity. The rates and patterns in the description of new species from the 1750s show no sign of approaching an asymptote and even accelerated in the 2010s after the advent of molecular approaches to species delimitation. Species recognition studies of (semi-)cryptic species hidden in morpho-species complexes suggest a weighted average ratio of about an order of magnitude for the number of species recognized after and before such studies. New evidence also comes from extrapolations of plant:fungus ratios, with information now being generated from environmental sequence studies, including comparisons of molecular and fieldwork data from the same sites. We further draw attention to undescribed species awaiting discovery in biodiversity hot spots in the tropics, little-explored habitats (such as lichen-inhabiting fungi), and material in collections awaiting study. We conclude that the commonly cited estimate of 1.5 million species is conservative and that the actual range is properly estimated at 2.2 to 3.8 million. With 120,000 currently accepted species, it appears that at best just 8%, and in the worst case scenario just 3%, are named so far. Improved estimates hinge particularly on reliable statistical and phylogenetic approaches to analyze the rapidly increasing amount of environmental sequence data.

BACKGROUND

In 1825, Elias Magnus Fries (1794–1878) predicted that the fungi would prove to be the largest group in the vegetable kingdom, analogous to the insects in the animal kingdom. Notwithstanding that fungi are not actually part of the plant kingdom, how right he has proved to be as the bicentenary of his prediction approaches. By the 1960s a few mycologists were speculating that there might be as many fungal as plant species, but almost no attempts to calculate estimates from the available data were made. As concern over the conservation of biodiversity in general grew in the subsequent decades, culminating in the signing of the Convention on Biological Diversity in 1992, more precise figures on species numbers of all kinds of organisms were required. A series of estimates of the number of fungi settled on figures ranging from 500,000 to almost 10 million species, with 1.5 to perhaps 5 million receiving most support among mycologists. A recent study even predicts up to a trillion species of microorganisms globally (1); how many of these are supposed to be fungi is not specified, but if this estimate holds true and only 1% of these were fungi, the global estimate of fungal diversity would be a thousand times higher than the current highest estimate of 10 million species.

Different extrapolation techniques have been used to arrive at global fungal species richness estimates, including publication rates of new taxa (2), plant:fungus ratios (3, 4) similar to plant:insect ratios first used in

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Erwin's famous study (5), quantitative macroecological grid-based approaches (6–8), methods based on environmental sequence data including plant:fungus ratios (9, 10), and ecological scaling laws (1).

This article provides updated background information on the number of described species and explores new evidence obtained from rates of species discovery, extrapolations from fungus:plant ratios, and molecular sequence data from environmental samples. We conclude by indicating where undescribed species are likely to be found and recommend a working number suitable for general use in estimates of global and regional species richness.

We focus here on the numbers of species in the kingdom *Fungi*, that is, inclusive of the *Cryptomycota* and *Microsporidia*, and also including lichen-forming fungi. We do not, however, consider other organisms that mycologists study as fungi, such as the fungal analogues *Mycetozoa* (as an unranked supergroup or in the kingdom *Protozoa*, phylum *Amoebozoa*) and *Oomycota* and *Hyphochytriomycota* (in the kingdom *Heterokonta* = *Straminipila*). However, we caution that some data sets and papers discussed in the following sections are based on fungi in the broad sense and thus include information from the analogue phyla. While readers should bear this in mind, the numbers of known taxa in these groups are relatively modest, so their occasional unavoidable inclusion will not materially affect the interpretations presented here.

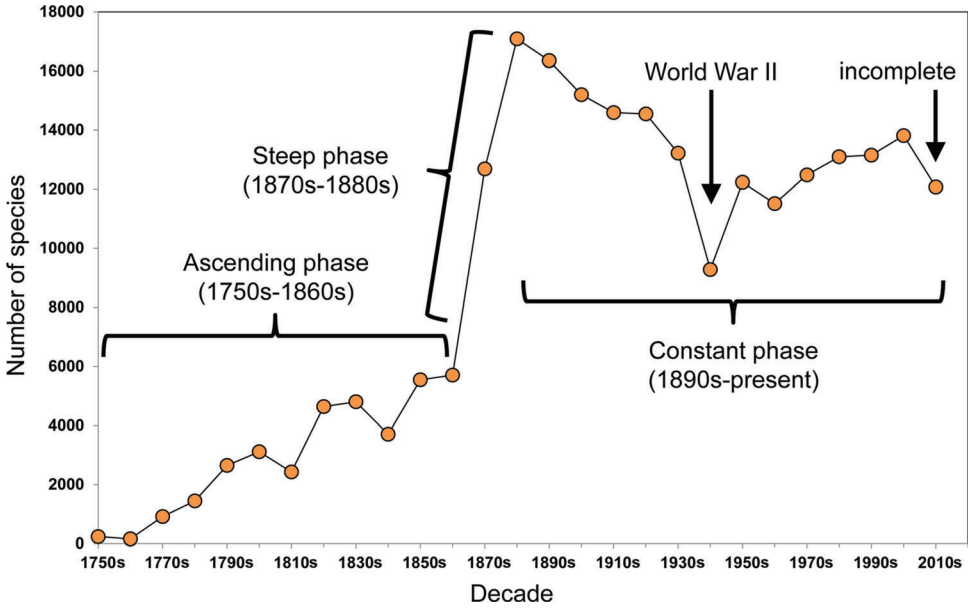
EVIDENCE FROM PUBLICATION RATES OF NEW TAXA

Numbers of Described Species

The number of new species described in each decade, as recorded in the Index Fungorum database (http://www.indexfungorum.org), shows three distinct phases (Fig. 1): an ascending phase in which progress was fast in the 1750s to 1860s, a steep phase as microscopy came into general use and there was intensive collecting in hitherto barely explored parts of the world in the 1870s to 1880s, and then a relatively constant phase from the 1890s to the present day. The partially higher figures in the first decades of the "constant phase" reflect continuing exploration and a number of prolific individual mycologists. The rate of description over the past 40 years has, however, remained fairly constant at around 1,300 per year (Fig. 2), with peaks generally relating to particular major monographic works.

Of special note is the somewhat steep increase in the rate since 2010 to around 1,800 per year. This resurgence in species description is largely attributed to the increasing use of molecular techniques for species delimitation and is of particular note because this increase followed, and even accelerated after, the ending of the separate naming of morphs of the same species in 2011 (when the provision for a Latin diagnosis or description was also eliminated). Prior to that date, the totals for annually described "species" included separate names given to different morphs of the same species.

FIGURE 1 Numbers of newly introduced species names of fungi for each decade from 1750 to 2010. Based on data from the Index Fungorum database provided by P. M. Kirk.



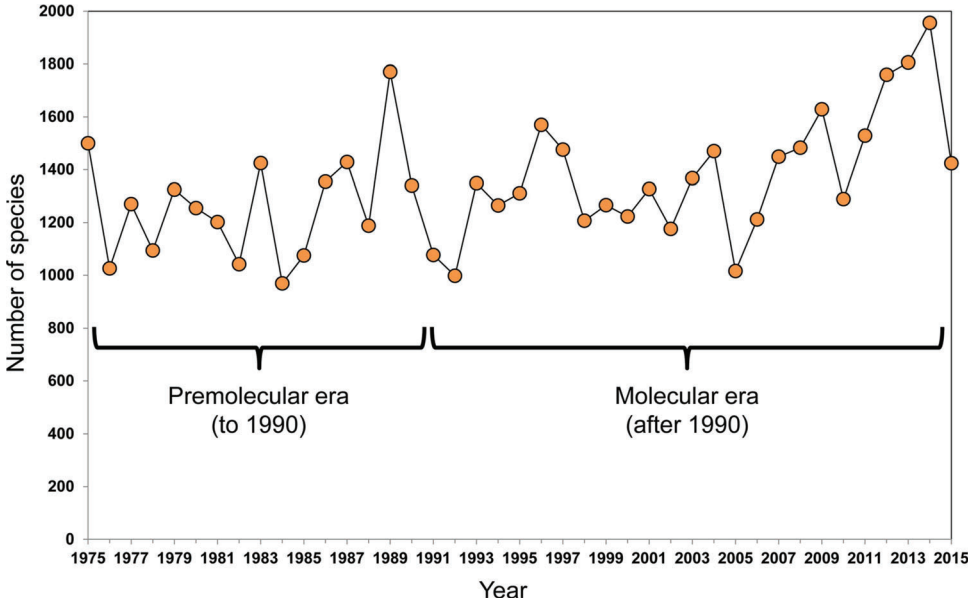


FIGURE 2 Numbers of newly introduced species names of fungi for each year from 1975 to 2015. Note that the data for 2015 were incomplete when this work went to press. Based on data from the Index Fungorum database provided by P. M. Kirk.

The number of newly named species does not, however, equal the number that have been truly described for the first time. It is much easier to name a fungus as new to science rather than to ascertain that it has previously been named. This is not simply a matter of comparing material to that of previously recognized species in the same genus but requires meticulous detective work, because not uncommonly, species were described in genera they do not belong to. Further, species concepts change, for example, in genera where morphologically identical fungi were regarded as different species if they occurred on different plant species. The number of accepted fungi can only be obtained by counting those recognized as "good" in each genus. This has been done since 1943 through the 10 editions of *Ainsworth & Bisby's Dictionary of the Fungi* and now annually through Species Fungorum inputs to the Catalogue of Life (http://www.catalogueoflife.org/annual-checklist/). An analysis of these data shows that the total number of existing species names runs at around 2 to 3 times that of currently accepted species (Fig. 3), a figure consistent with the ratio of 2.6 derived from an analysis of names in monographic works (11). The number of described good fungal species currently stands at around 120,000.

Unlike the situation in many other groups of organisms (12), the number of good fungal species continues to rise steeply decade by decade, with no indication of leveling off. The steepness of the curve would be even greater if there were more mycologists actively involved in species discovery, using either morphological or molecular approaches.

The number of species being recognized is necessarily impacted by the species concepts used. While in the premolecular era, species circumscriptions were based almost entirely on morphological and sometimes biochemical and cultural features, the incorporation of molecular information has led to a refining of species concepts. There is, however, a plethora of species concepts in biology (13, 14) and no single objective criterion that can be applied universally. Because the purpose of names is to provide a means of communication, a pragmatic species concept is essential, which may be defined as the following: "a species is what it is useful to give a species name to." (15). There have been numerous attempts to apply mechanistic systems for species recognition in phylogenetic trees, but the consolidated species concept has much to commend it and ultimately provides a broad consensus on how to recognize fungal species. The consolidated species concept adopts a polyphasic approach combining morphological, ecological, and phylogenetic species concepts (16) and is being increasingly taken up by mycologists. While in some situations this may lead to more species being recognized than otherwise would have been the case, in others it results in formerly separately distinguished species being united. Therefore, over- and underestimations in particular cases are usually balanced out. A rarely considered notion is the time scale: a proportion

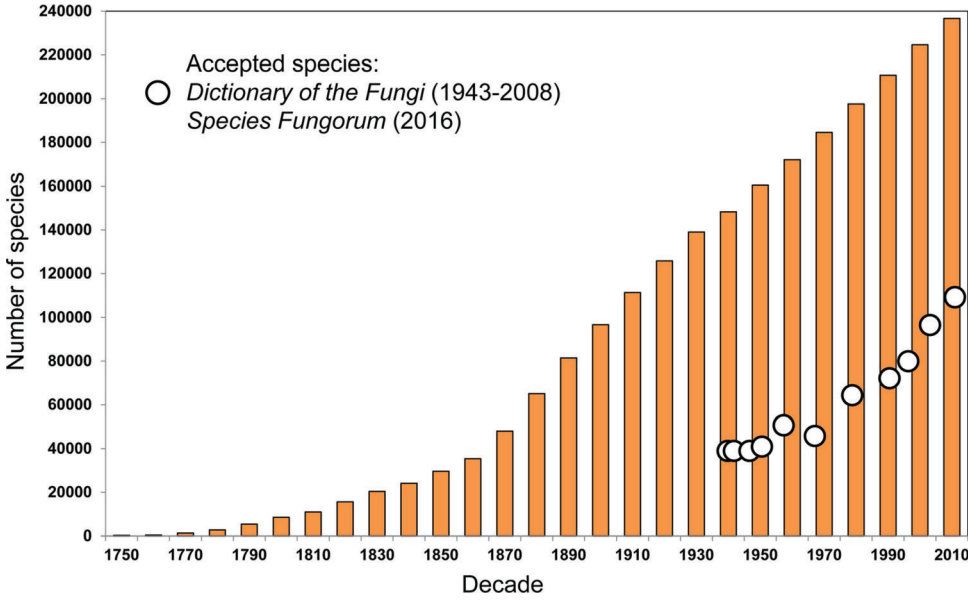


FIGURE 3 Growth in the total catalogued number of species names of fungi by decade from 1750 compared with the global number of accepted species. Based on figures adopted in the 10 editions of *Ainsworth & Bisby's Dictionary of the Fungi* for 1943–2008 and data in the Index Fungorum and Species Fungorum (Catalogue of Life) databases provided by P. M. Kirk.

of "species" is in active speciation, with incomplete lineage sorting, yielding it difficult to resolve good species (17); in such cases, determining the number of species is philosophical, since that number is in active flux. A large number of new fungal species continue to be described with no molecular data, either due to the age of the underlying material (e.g., unveiled in older collections) or by taxonomists who do not have access to molecular techniques; assessing the proportion of good species in such taxa is therefore difficult.

Patterns in Taxon Discovery

Some predictable patterns in the recognition of published taxa in the rank of genus and above have been recognized and applied to a broad set of organisms, including fungi (2). By combining the full classifications of around 1.2 million species of all groups of organisms, these authors found, as might have been expected, that accumulation curves of higher taxa over time showed that these were more completely described and in many cases approached an asymptote. The use of the higher taxon approach was then validated by comparing the actual number of known species with that predicted from the accumulation curves. The authors then fitted asymptotic regression models to the actual curves for different groups to provide estimates of undiscovered species numbers. In the case of fungi, this led to a total number of expected fungal species of 611,000 (standard error ± 297,000). This conclusion was, however, misleading, because the analysis was based on the assumption that there were only 43,271 catalogued fungal species, rather than the nearly 100,000 accepted at that time (18). Intriguingly, a recalculation using their method with the latter species number generates a figure of around 1,400,000—not far out of line with estimates derived from extrapolations based on plant:fungus ratios (see below).

EVIDENCE FROM SPECIES RECOGNITION STUDIES

Unknown fungi are now considered to come mostly from two sources: (i) newly discovered species by means of traditional inventory methods in little-studied areas and habitats and (ii) newly discovered lineages through environmental sequencing. A further, neglected but significant source of unrecognized fungal diversity is the restudy of known taxa, applying species recognition techniques to molecular data, using either the fungal barcoding locus ITS (19) or multilocus approaches. Exemplar taxa are the fly agaric, *Amanita muscaria*, and the golden chanterelle, *Cantharellus cibarius*, which contain numerous, often regionally distributed species (20, 21). The record is currently set by the basidiolichen fungus

Cora glabrata, which has been shown to contain at least 189 species (6, 22). We surveyed 45 such studies published between 1998 and 2016, including studies of non-lichenized and lichenized forms, and computed the ratio of species recognized in the target group before and after the study (Table 1). Most studies started out with a single or few species, whereas two looked at complexes of up to 18 species—*Protoparmelia* (23) and *Usnea* (24)—and one revised a complex of 71 species accepted prior to the study, in the genus *Coprinellus* (25). The posterior:prior ratios in the number of species recognized ranged from 0.67 (from 18 to 12 species in a group of *Usnea* [24]) to 189 (from 1 to 189 species in the basidiolichen *Cora glabrata*) (6, 22). Other high ratios were found for *Dictyonema* with 59 (26), *Cladosporium cladosporioides* with 22 (27), *Sticta weigelii* with 15 (28), *Microbotryum violaceum* with 11 (29), and *Fusarium graminearum* with 7 (30). Environmental sequence and genomic data revealed high ratios for the genera *Archaeorhizomyces*, with 145 (31), and *Cyphobasidium* with 26 (32), as well as for the still barely explored *Cryptomycota*, with 138 (33, 34), in which the true figure could be much higher. The median ratio over all evaluated studies was 3.0, and the arithmetic mean was 14.5 (Table 1).

To obtain a more reliable picture, we adjusted these ratios by geographic area covered. If a species complex had a global distribution prior to the study, and if based on material from a single geographic area, it was found to represent several species with presumably restricted distribution, the detected ratio was adjusted by the factor 2 (expanding to both hemispheres in cases in which one hemisphere was covered), the factor 3 (for tropical fungi for which a single tropical region was covered), and up to the factor 6 (for cosmopolitan fungi for which a single region within a single continent was studied). These factors were derived from selected global studies, for example, in the genera *Cladosporium* (27), *Cora* (6, 22), *Fusarium* (30), and *Sticta* (28). In some cases, we added unpublished data to the adjusted values; thus, for *Archaeorhizomyces* we currently recognize at least 500 species (B. E. Smith and R. Lücking, unpublished data), and for *Cyphobasidium*, we recognize about 100 species from deposited GenBank sequences (32). The thus adjusted ratios ranged from 0.7 (*Usnea*) to 435 (*Archaeorhizomyces*), with a median value of 10.5 and an arithmetic mean of 39.3.

To predict the global species richness of *Fungi* using these raw and adjusted ratios, we employed two further adjustments. First, we weighted the ratios according to the number of species recognized in a genus in the 2008 *Dictionary of the Fungi* (18). For instance, a genus in which a single species had been recognized received a low weight (1.0), since even a high number of newly recognized species will only contribute minimally to global fungal species richness. This adjustment avoided a high impact of extraordinary ratios such as in the previously monotypic genus *Cora*. In contrast, for groups in which a large number of species had already been recognized, the ratios based on a study of one or a few species were upweighted, e.g., in the genus *Puccinia* (35), with 4,000 accepted species. In addition, to account for ecological bias in the evaluated studies, we applied a group weight according to the major lifestyle attributed to each genus; to that end, we divided the approximately 100,000 known fungi in 2008 as follows: 40,000 saprotrophic (weight 4.0), 20,000 phytopathogenic (weight 2.0), 20,000 lichenized (weight 2.0), 8,000 ectomycorrhizal (weight 0.8), 5,000 endophytic (weight 0.5), 3,000 entomopathogenic (weight 0.2), 2,000 aquatic (weight 0.2), 500 endomycorrhizal (weight 0.05), 500 marine (weight 0.05), 400 soil (weight 0.04), and 100 human pathogenic (weight 0.01). The categories aquatic, marine, and soil are used here for cases where the habitat is known but not the biology. These proportions denote estimates based on known fungi in 2008, not estimated proportions of extrapolated global species richness, in which, presumably, categories such as plant pathogens and soil fungi will have much higher values. Using these weights, the overall arithmetic mean of the posterior:prior ratio for all evaluated studies amounted to 11.3. This suggests that even without including important sources of new species discoveries, the already accepted *Fungi* may contain up to 10 times as many species as currently recognized, resulting in more than 1 million estimated species. Remarkably, this is about 4 times higher than the currently estimated synonymy ratio of 2.6, suggesting that a large proportion of these unrecognized species have no names available.

EVIDENCE FROM EXTRAPOLATIONS BASED ON PLANT:FUNGUS RATIOS

The widely cited and accepted plant:fungus ratio-derived global estimate of 1.5 million species of fungi was based on information from several independent sources (3): (i) the numbers of fungi reported from all habitats in the United Kingdom and sites within the United Kingdom compared with the number of plant species present, (ii) the numbers of host-specific fungi on particular plant species, (iii) percentages of new species being discovered, and (iv) extrapolations to the global level based on conservative estimates for the number of

TABLE 1 Selected species recognition studies in different groups of fungi

Taxon	Genus[a]	Lifestyle	Coverage[b]	Range[c]	Ratio[d]	Factor[e]	Adjusted[f]	Weight[g]	Reference
Acantholichen pannarioides	1	Lichenized	Neotropical	Neotropical	6	1	6	8	100
Amanita muscaria	500	Ectomycorrhizal	N. Hemisphere	Global	6	2	12	6,000	20
Archaeorhizomycetes	0	Soil fungus	N. Hemisphere	Global	145	3	500	500	31
Aspergillus flavus	266	Saprotrophic	Australia	Global	2	6	12	3,192	101
Aspergillus fumigatus	266	Human pathogenic	Global	Global	2	1	2	532	102
Auxarthron zuffianum	15	Saprotrophic	Global	Global	2	1	2	30	103
Beauveria	9	Entomopathogenic	Global	Global	4	1	4	36	104
Blastomyces dermatitidis	1	Human pathogenic	N. America	Global	2	1	2	2	105
Botrytis cinerea	54	Phytopathogenic	Europe	Global	2	6	12	648	106
Calonectria pauciramosa	34	Phytopathogenic	Global	Global	3	2	6	204	107
Cenococcum geophilum	1	Ectomycorrhizal	N. America	Global	5	6	30	30	108
Cladosporium cladosporioides	150	Saprotrophic	Global	Global	22	1	22	3,300	27
Coccidioides immitis	3	Human pathogenic	N. America	N. America	2	1	2	6	103
Coniophora puteana	20	Saprotrophic	Global	Global	3	1	3	60	109
Coprinellus	100	Saprotrophic	Global	Global	1,2	1	1.2	120	25
Cora	1	Lichenized	Subglobal	Global	189	1	189	189	6, 22
Corella	0	Lichenized	Central/S. America	Central/S. America	11	1	11	11	26
Cryptomycota	0	Aquatic	N. America	Global	39	6	234	234	33
Cyphellostereum	1	Lichenized	Central/S. America	Global	15	3	45	45	26
Cyphobasidium	0	Lichenicolous	N. America	Global	26	4	100	100	32
Dictyonema	6	Lichenized	Subglobal	Global	59	3	177	1062	26
Fusarium graminearum	111	Phytopathogenic	Global	Global	7	1	7	777	30
Grosmannia clavigera	1	Phytopathogenic	N. America	N. America	2	1	2	2	110
Hymenoscyphus albidus	155	Phytopathogenic	Europe	Europe	2	1	2	310	111
Lasiodiplodia theobromae	9	Phytopathogenic	Pantropical	Pantropical	3	1	3	27	112

Letharia	2	Lichenized	N. Hemisphere	N. Hemisphere	3	1	3	6	113
Lobariella	5	Lichenized	Neotropical	Neotropical	3.8	1	3.8	19	114
Metarhizium anisopliae	9	Entomopathogenic	N. America	Global	2	6	12	108	115
Microbotryum violaceum	87	Phytopathogenic	Europe/N. America	Global	11	4	44	3,828	29
Neofusicoccum parvum/ribis	13	Phytopathogenic	Regional/host	Global	2	60	150	1,950	116
Paracoccidioides brasiliensis	1	Human pathogenic	S. America	S. America	4.0	1	4	4	117
Parmelia	95	Lichenized	Europe/N. America	N. Hemisphere	3	1.5	4.5	428	118
Parmelia saxatilis	1	Lichenized	N. America	Global	4	3	12	12	119
Parmotrema reticulatum	348	Lichenized	Subglobal	Subglobal	4	1	4	1,392	120
Phialocephala fortinii	1	Endophytic	Europe	Global	7	6	42	42	121
Protoparmelia	20	Lichenized	Global	Global	1.6	1	1.6	32	23
Puccinia monoica	4,000	Phytopathogenic	N. America	Global	1.8	6	10.5	42,000	35
Rhizoplaca melanophthalma	11	Lichenized	N. America	Global	2.5	6	15	165	122
Stachybotrys chartarum	44	Saprotrophic	N. America	Global	2	1	2	88	123
Sticta fuliginosa	114	Lichenized	Global	Global	15	1	45	5,130	28
Sticta weigelii	114	Lichenized	Global	Global	23	1	23	2,622	28
Strobilomyces	20	Ectomycorrhizal	Asia	Global	3.5	6	21	420	124, 125
Tricholoma scalpturatum	200	Ectomycorrhizal	Europe	Global	2	6	12	2,400	126
Uncinocarpus reesii	3	Saprotrophic	Global	Global	2	1	2	6	103
Usnea	338	Lichenized	N. Hemisphere	N. Hemisphere	0.7	1	1	338	24

[a]Number of species recognized in the corresponding genus in reference 18.
[b]Origin of material used in the study.
[c]Global distribution of the group.
[d]Ratio of the number of species recognized after versus before the study.
[e]Geographic adjustment.
[f]Adjusted ratio based on raw ratio and geographic adjustment.
[g]Weight based on adjusted ratio and original number of species recognized in the corresponding genus in reference 18 (in some instances [*Acantholichen*, *Sticta*, *Usnea*] further adjusted based on unpublished data).

plant species, making allowances to discount separately named morphs of the same species. The 1.5 million figure was considered conservative because, while numbers of fungi from all sources, including organisms other than plants and soil, were included in the species totals for particular places, no allowances were made for undiscovered fungi in and on the millions of insects predicted to exist.

Some figures used in these early extrapolations have subsequently been revised upward. Ten years later, it was pointed out that the number of plant species and the fungus:plant ratio were too conservative and the vast number of insect fungi had not been considered. As a consequence, some revised estimates ranged between 2.7 and 9.9 million fungal species (4).

The total inventory of fungal species found in field surveys in a particular site continues to increase year by year, even with regular visits spanning several decades, while the number of plant species remains more or less unchanged. Esher Common (Surrey, United Kingdom) is by far the most investigated site by field mycologists in the world, and to date about 3,400 species have been found (B. M. Spooner, personal communication). Because the number of vascular plant species remains at 420, this now gives a fungus:plant ratio of 8:1.

Some preliminary results from next-generation sequencing of soil samples from a wood within the second most field-surveyed site in the world, the Slapton Ley National Nature Reserve in southwest England, are illuminating in relation to the fungus:plant ratios (G. W. Griffith, B. S. Dentinger, and D. L. Hawksworth, unpublished). The wood studied has 1,136 species of fungi and 88 vascular plant species recorded during field surveys, a ratio of 12.9:1. However, just 686 sequence-based species (accounting only for taxa represented by five or more sequences) were recovered from soil samples (and a further 153 taxa were represented by one to four sequences). What is intriguing is that of the 164 named genera represented in the next-generation sequencing data set, only 32 were among the 549 genera collected in the fieldwork, which included fungi on plant parts and plant litter, not just sporophores emerging from the soil. The actual ratio in a site may therefore be substantially higher than indicated by traditional inventory techniques.

The interpretation of some data sets needs to be considered in the light of such long-term investigations. Ratios comparing fungal sequences recovered from environmental samples with plants growing on the sample sites have given some surprising ratios. For example, data from a forest in North Carolina gave fungus:plant ratios of 19:1 (491 fungi and 26 plants) and 13:1 (616 fungi and 48 plants), leading to a suggestion that there may be 3.5 to 5.1 million fungal species worldwide (9). A more modest ratio of 7.5:1 (1,500 fungi and <200 plants) was obtained from boreal forest soils in Alaska (36). In contrast, Tedersoo et al.'s (37) impressive study of 365 globally distributed soil samples using metabarcoding molecular methods (see below) led the authors to conclude that fungal species richness had actually been overestimated by 1.5 to 2.5 times from that based on plant:fungus species ratios. Any such extrapolation from soil samples alone to the total fungal biota at a site is, however, unsound as evidenced by the above data from the Slapton reserve. Much of the actual fungal species diversity in a site will inevitably be missed in soil sampling, because most is above ground, on and in plants and animals of all kinds, in water, in lichens, on rocks, etc.

Collections made in brief smash-and-grab collecting trips or even extending over several years will only start to reveal what fungi may actually be present at a site. Studies such as that of Piepenbring and collaborators (38), in which 567 species of fungi and 311 species of plants were found along a 500-m pathway in Panama over 2 years, giving a ratio of just 1.8:1, have to be interpreted in this context. The ratio resulting from that study would be expected to rise significantly if the period over which it was conducted had been 2 decades rather than 2 years, and with increased attention to microscopic fungi.

When considering fungi of all biologies and in all ecological niches in a site, the ratio can be expected to vary depending on the geographical location, as recognized some 25 years ago (3). It is not surprising, therefore, that the studies of fungus:plant ratios in a site, obtained by field survey and molecular approaches, have generated ranges from 1.8 to 19.1:1. The mean of the figures cited in this section yields a ratio of 9.8:1, suggesting that the ratio of around 6:1 arrived at in 1991 may be conservative. With the currently accepted number of approximately 380,000 vascular plant species (39), this adjusted ratio would predict a number of nearly 3.8 million fungal species.

EVIDENCE FROM ENVIRONMENTAL SEQUENCING TECHNIQUES

The advent of obtaining taxon-specific, molecular sequence data from environmental samples, most commonly soils but also water and plant tissues, has generated a new source of data for estimating global

species numbers. It is commonly found that numerous sequences thus obtained do not have any matches with those from named fungi in GenBank (see below), and this has led to speculations that previous estimates have been too low.

Generalizing data from environmental samples is problematic for several reasons, including that the broader geographic context of detected operational taxonomic units (OTUs) is usually unknown. For example, using 454-sequencing technology, two 0.25-g soil samples from an Alaskan forest collected just 1 m apart had only 14% of fungal species in common (36). In a pyrosequencing study in the Andes, 1,839 species-like taxa were found, of which 25% were most similar to other unidentified environmental samples, with significant differences between forests at different altitudes (40). The most comprehensive study of soil samples to date is that of Tedersoo et al. (37), who analyzed 2-g soil samples from pooled soil in 5-cm deep cores taken from 365 global sites. They obtained 80,486 fungal OTUs, used a 98% sequence similarity for species recognition, and did not consider almost half in further analyses because they were singletons and potentially artifacts; in that study, plant:fungus richness declined toward the poles. However, in concluding that extrapolations from plant:fungus ratios were unjustified (see above), the authors did not take into account fungi other than those in soil, such as those in and on aerial parts of plants, in decaying vegetation, lichen-fungi, and entomogenous fungi.

Especially instructive is a study of 928 swabs of dust samples across the continental United States by direct PCR and using high-throughput sequencing (HTS) to analyze them (41); not only were 38,473 fungal taxa detected, but there were clear geographic patterns with a predictive value of placing a sample within 230 km. Of the 38,473 taxa, however, sequences of about 40% could not be matched with any named species (R. R. Dunn, personal communication).

Just how many unidentified sequences generated from environmental samples represent undescribed species is uncertain. Compared with the 120,000 formally recognized species (see above), as of 25 November 2016, there were only 34,878 named fungal species in GenBank, but there were a further 94,059 species-level OTUs with no names (C. L. Schoch, personal communication); these figures have increased by 68% and a staggering 117%, respectively, in the past 5 years. However, only a minute fraction of environmental sequences are deposited individually in GenBank; the vast majority is placed in the Sequence Read Archive (SRA) (42). Currently, the SRA, on the query "(fungal OR Fungi) AND (internal transcribed spacer [or, ITS] OR ITS1 OR ITS2)," returns 179 studies, 1,822 biosamples (i.e., environmental samples), and 14,334 experiments or HTS runs, containing 928 million fungal ITS reads, with an average length of 353 bases (SRA: https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=search_obj; accessed 12 March 2017). In contrast, using the same query, GenBank (43) returns 993,987 sequences obtained predominantly through Sanger sequencing (GenBank: https://www.ncbi.nlm.nih.gov/genbank; accessed 12 March 2017). Thus, at present there are almost 1,000 times more HTS reads than Sanger sequences. Only 3 years ago, this ratio was 18:1, which means it has increased by a factor of more than 50 in this short period.

Obviously, a proportion of the unidentified sequences can be expected to represent known but as yet unsequenced species, especially considering that about 85,000 of the currently accepted fungal species have no sequence data available. In the case of *Mortierella*, a reference set of type and other strains was sequenced and then compared with unnamed sequences in GenBank (44); the authors modeled the effects of increasing type strain sequencing and found a linear relationship with the number of strains that could be identified, and they predicted a number close to that of the already described species.

One of the most significant problems with using environmental sequence data to extrapolate global fungal species richness is the approach to define OTUs. Due to the volume of data generated and the usual broad taxonomic range detected, alignment-based methods that would allow us to critically define species as phylogenetic lineages are impossible to use as routine techniques. Thus, environmental sequence data are analyzed using clustering techniques, and OTUs as equivalents to species are defined on thresholds, ranging between 95 and 99% depending on the study. This approach has several shortcomings. First, species do not exhibit fixed sequence thresholds; in contrast, sequence divergence and hence the barcoding gap depend on the evolutionary age of the species complex. Second, commonly used thresholds of 95 to 97% are unrealistic. Assuming that the ITS barcoding locus has a length of, e.g., 500 to 600 bases, 5% divergence would correspond to 25 to 30 bases. In most studied species, infraspecific ITS variation is much lower, with an average of about 1%. Theoretically, a more realistic threshold of 99% would greatly increase the number of OTUs detected in a data set. However, this effect is more than outweighed by the sensitivity of clustering methods to variation in the data, including the common CAFIE (carry-forward-

incomplete-extension) sequencing error common to all base flow technologies (45) (see below).

This error and other problems with clustering techniques can lead to an overestimation of the number of OTUs at several orders of magnitude, which may result in serious bias when using such estimates for global extrapolations. We tested this effect with SRA data of the soil fungus *Archaeorhizomyces* (Smith and Lücking, unpublished). Through a blast script, we obtained 106,563 ITS reads from the SRA that were reasonably close to the type species, *Archaeorhizomyces finlayi*. Clustering analysis using USEARCH (46) resulted in the following numbers of OTUs depending on the set threshold: 2,658 at 95%, 5,793 at 97%, 10,630 at 98%, and 28,435 at 99%. Thus, the difference between the 95% and the 99% threshold is an order of magnitude. Alignment-based phylogenetic analysis suggests the presence of approximately 500 species-level lineages in this data set. Thus, depending on the threshold level used for species-level separations, clustering could overestimate the actual species richness in this case by a factor of between 5 and 50.

Lücking and colleagues (45) showed that severe problems with estimating OTUs through clustering emerge from even minor sequencing errors. The CAFIE error is common to all next-generation sequencing technologies, because it represents a statistical error of the distribution of bases among wells on a plate through a given base flow. Through careful adjustment, this error can be held to a minimum of less than one erroneously phased insertion per read, i.e., about 0.3% assuming an ITS1 or ITS2 read length of approximately 300 bases. This proportion is well within the expected infraspecific variation even at a high level of 99% similarity and thus theoretically should disappear as background signal. However, the random distribution of this error across the entire sequence, which cannot be recognized by quality filtering methods, especially not in environmental samples where no expected sequence patterns exist for comparison, causes clustering methods to interpret these differences as taxonomic, leading to serious overestimations by distribution sequences of the same taxon, but with different minor errors, into separate clusters. This is because clustering methods work with local alignments of small, highly similar sequences, whereas phylogenetic methods require alignment of sequences of a data set to template sequences obtained from Sanger sequencing. As a consequence, in a broad alignment including Sanger reference sequences, erroneous and phased indels will be located in mostly gapped columns, which have practically no influence on the resulting topology, whereas in clustering methods, they will frequently be aligned with non-homologous base calls and falsely interpreted as substitutions (45).

In the above (45), a proportion of less than 1% erroneously phased indels caused by CAFIE errors in the ITS of a single species caused overestimation of the number of taxa by a factor of 35 at a 95% threshold level, 137 at 97%, and 980 at 99%. Thus, instead of disappearing as background signal, the sequencing error had a tremendous effect on OTU evaluation when in that case it was known that all sequence data came from a single target species. Even after removing all erroneous indels, clustering continued to overestimate species richness by a factor of 4 at a 95% threshold level, 8 at 97%, and 116 at 99%, due to minor remaining infragenomic variation and other sequencing errors. In contrast, alignment-based phylogenetic analysis of a broad set of nearly 2,000 reads recovered a single species, even with all sequence errors included. Thus, while environmental sequencing continues to accumulate a vast amount of important data on unrecognized fungi, extrapolations from these data must be taken with great care when based on clustering methods. An extreme example is a recent study which predicts up to a trillion species of microorganisms globally based on OTUs derived from environmental sequence data (1), using ecological scaling laws which, put in simple terms, predict how the global, limited number of N individuals would divide into S species, taking into account resource competition based on body size and other factors. This number, while certainly intriguing, appears to be exaggerated, and it would be interesting to see what alternative prediction would be obtained when defining the underlying taxonomic entities in this analysis through alignment-based phylogenetic methods.

In spite of these shortcomings, it is clear that environmental sequencing is now the major source of discovering novel fungal taxa, at least in the same order of magnitude as taxa recovered in species recognition studies or perhaps much higher. It follows that to properly catalog these fungi, a naming system for voucherless sequences must be developed, and proposals on how to do that are currently under consideration (47–51).

In addition to discovering new species, environmental sequencing may reveal unknown higher fungal taxa up to the class level. The best-known example is the recently discovered class *Archaeorhizomycetes* (31, 52). In Tedersoo and colleagues' study (37), ~6% of all fungal OTUs below the phylum level could not be assigned to any known class. Further clustering of unidentified fungal sequences at 70% sequence similarity

revealed 14 distinct taxonomic groups comprising >7 OTUs, suggesting that there are several deeply divergent class-level fungal lineages that have not yet been described or previously sequenced. A follow-up study by the same group (53) also revealed numerous new lineages at least at the order level.

WHERE ARE THE UNDESCRIBED FUNGI?

If there are at least 1.5 million or more fungi on Earth, and we know of just 120,000, where can the missing 1.38 million species be? As outlined, three major sources for unrecognized fungal diversity seem to emerge: (i) geographic areas and ecological habitats that are largely understudied, particularly in tropical regions and biodiversity hot spots, (ii) ecologically cryptic fungi that occur in the environment but usually do not manifest themselves via discernible structures other than microscopic hyphae and mycelia, and (iii) so-called cryptic species hidden under well-established names.

Biodiversity Hot Spots

It is generally recognized that missing species of all groups of organisms will be found in biodiversity hot spots (54). A novel quantitative method to identify hot spots of unrecognized species richness is the grid approach (6–8), which relies on linear interpolation and hence gives more reliable estimates than nonlinear extrapolation. The area in question is divided into grids, and observed richness per grid is linearly correlated with predictive macroenvironmental parameters and sampling effort, using at least one well-sampled grid for calibration. This method can be applied to both traditional inventories and data based on environmental sequencing, as long as species are reliably delimited and macroenvironmental predictors can be reasonably derived from the data. Thus far, the method has been employed for lichen-forming fungi in the families *Graphidaceae* and *Trypetheliaceae* and the genus *Cora*, resulting in estimates ranging between 1.5 and 4 times the number of known species.

South America is generally recognized as having major hot spots, and various studies point to high levels of novelty there. By combining data from basidiomes and sequence data from ectomycorrhizal roots in a *Dicymbe corymbosa* forest in Guyana, Henkel and collaborators (55) estimated that over 250 species were associated with this one tree species in this single site. López-Quintero and colleagues (56) found that of 632 macromycete species in Colombian Amazonian forests, 52% could not be identified to the species level, a significant proportion likely representing undescribed species. Truong and coworkers (57) collected 1,430 basidiomes during just four collecting expeditions in southern South American *Nothofagaceae*-dominated forests; they generated 439 OTUs out of 957 specimens, of which 308 (ca. 70%) did not match any in the UNITE dynamic database at 97 to 99% similarity and thus did not correspond to any "species hypothesis" currently in the database.

Little-Explored Habitats

There are an enormous number of potential sites for fungi in any locality, and in the case of the tropics at least 31 ecological niches meriting study can be recognized, many requiring different techniques and specialists to explore (58). While no site on Earth can yet be considered to have a complete inventory of the fungi present, some habitats stand out as particularly underexplored.

The situation can be exemplified by the fungi which obligately grow on lichens, the lichenicolous fungi. With a few notable exceptions, this niche was largely overlooked by lichenologists and other mycologists until the mid-1970s. In 1976, there were just 457 species known worldwide (59), but by 2016 that number exceeded 1,800 (http://www.lichenicolous.net/); that represents a 394% increase over 40 years, or 98.5% per decade. This growth is reflected at the more local level, where in Great Britain and Ireland the number known rose from just 218 species in 1983 to 403 in 2003, an increase of 85% over 20 years (60); the number has now passed 500 and continues to rise (D. L. Hawksworth, unpublished). That figure compares with around 1,900 lichenized species in the same region, implying by extrapolation to the global scale that there may be as many as 25% as many lichenicolous fungi as lichenized species, i.e., around 5,000 species. This hypothesis is supported by studies of these fungi in areas where they have not previously been investigated; for example, of 189 species reported from Isla Navarino in Chile on the basis primarily of collections made in just 1 year, 6 genera and 60 species (32%) were new to science (61). And these figures do not include the so-called endolichenic fungi that can be cultured from lichen thalli or are only known from sequence data (e.g., 62, 63). In addition, emerging studies suggest the presence of highly specific, yet morphologically cryptic, basidiomycete yeasts and other basidiomycetes in the cortex of lichen thalli, which add yet another dimension to unknown fungal diversity (32, 51).

The experience with lichenicolous fungi appears or can be expected to be paralleled in other little-explored

habitats, including bryophytes (64–66), algae (67, 68), endophytic fungi inside vascular plants (69–71), tropical foliicolous and fungicolous fungi (72), mammal guts (73), insect guts (74–77) and exoskeletons (78, 79), on and in rocks (80, 81), and in deep sea and ocean sediments (82), to name just a few.

Cryptic Species

Biologically distinct species which are morphologically indistinguishable, so-called cryptic species, were recognized 2 decades ago as one place where some of the predicted "missing fungi" might be discovered, since physiological and other biological divergence evidently often precedes morphological divergence in the evolution of fungal species. Based on studies of selected complexes then available, it was suggested that the number of known fungi might rise by a factor of 5 or more for this single reason (83). Molecular studies reveal that cryptic speciation is widespread through diverse groups of fungi, including plant pathogens, clinically important fungi, lichen-forming fungi, and mushrooms. Recognition of such cryptic speciation is indeed playing an increasingly important role in species discovery, as outlined above, and the ratio derived from current studies, 11.3:1, is about twice as high as estimated by Hawksworth and Rossman (83).

Existing Reference Collections

It has been estimated that over half of the projected 70,000 undiscovered plant species have already been collected and await description (84). Similarly, most reference collections of fungi, whether of dried specimens (fungaria) or living cultures (culture or microbial resource collections), include material that is not named to the species level, and in some cases not even to the genus level. Further, some collections and isolates referred to named fungi not uncommonly prove, on critical examination, to be different species. It has been suggested that around 20,000 fungal species have been collected but are as yet undescribed (83), but that is likely to be an underestimate because many mycologists have large backlogs of material awaiting formal description.

One of the challenges in the linking of newly obtained sequence data with named material in collections, particularly those that are type material, is fixing the application of the species name. Currently, the 34,878 species with sequences in GenBank (see above) represent just 29% of the 120,000 known species. Obtaining sequences for the missing 71% of named accepted species is essential to determine the novelty of recovered sequences from the environment, specimens, or isolates. While some researchers have had considerable success in recovering DNA from dried specimens (85), even from a dried *Hygrophorus* basidiome collected as far back as 1794 (86), this is not always possible (87). Aged dried material generally exhibits high levels of DNA degradation, making access through conventional extraction and PCR methods difficult (88, 89). In view of the serendipity of obtaining satisfactory results, collection curators are often unwilling to allow parts of often scant, irreplaceable type specimens to be destroyed for DNA extraction. Several attempts are now being made at using high-throughput sequencing technologies to obtain sequence data from highly fragmented DNA (88). One of the main problems in this respect is contamination, since even for highly variable loci, it will be a challenge to piece small fragments together without the risk of generating artificial chimeras. Therefore, efforts should be made to identify highly diagnostic regions in the ITS or other loci that allow the identification of a taxon even when only short fragments are at hand (89), as exemplified by comparing the ITS1 and ITS2 subregions (90).

There is a particular problem regarding the type species of generic names. An impressive 5,317 generic names of fungi are represented in GenBank (C. L. Schoch, personal communication), but in many cases the sequences are not from the name-fixing type species. A concerted attempt has been initiated to sequence the type species of genera currently not represented in DNA databases. This involves recollection and/or isolation from the geographical area of the original material and from the same host or substrate when no DNA has been recovered from the type material (91). Sequenced material of species can then in some cases be designated as an interpretive type—an epitype (92). Such an effort involves global collaboration of sequencing laboratories with colleagues in countries where high unknown diversity is located.

TOWARD A WORKING NUMBER OF GLOBAL FUNGAL SPECIES RICHNESS

There is general acceptance among mycologists that the global number of fungal species is a seven-digit figure, in the range 1 to 5 million (10), with "at least" 1.5 million now predominating, though a possible order of magnitude higher has been hinted at (93) and a minimum figure of 611,000 to 712,000 was arrived at (2, 94), leaving aside the figure of potentially billions of fungi extrapolated by Locey and Lennon (1). A recalculation of the number computed via Mora and colleagues'

approach (2) gives a figure of about 1.4 million (see above), close to the 1.5 million estimate which has come to be widely accepted by other biologists (54, 95–97), notwithstanding a few estimates that fail to appreciate the difficulty of inventorying fungi (98) or that equate numbers detected in soil alone as indicative of the total number at a site (37).

Unfortunately, the different techniques used to estimate global species richness are in part additive and in part overlapping or redundant, so that a combination of estimates from these studies is difficult. Thus, the revised estimate of nearly 3.8 million fungal species based on an updated fungus:plant ratio of 9.8:1 and 380,000 vascular plant species should include fungal species of all sorts with all types of ecologies and detected by different methodologies, including environmental sequencing and species delimitation methods. This number should then be congruent with approaches such as publication rates or scaling methods, but these approaches result in widely disparate estimates. Alternatively, an additive approach would be based on the three major sources of unrecognized fungal species richness, which can be reasonably sorted in descending order into (i) environmental sequencing, (ii) cryptic speciation, and (iii) other novel discoveries through traditional field work. For cryptic speciation, the above survey of established literature results in a weighted ratio of about an order of magnitude, i.e., more than 1 million additional species if based on 120,000 previously known species. If novel species from environmental sequencing are at least as high in number, this would add at least another million.

We conservatively assume that other novel discoveries (excluding species delimitation methods and environmental sequencing) will occur at a proportion of at best 10% of the latter two approaches. This assumption is based on the rate of 1,300 newly described species per year until about 2010, before the onset of rigorous species delimitation methods and environmental sequencing (see above). At this rate, it would take nearly 100 years to double the number of known species. Thus, while environmental sequencing and species delimitation methods would contribute about 2 million new taxa, other novel discoveries would add at best another 120,000 taxa within a reasonable time frame, for a total of a little over 2.2 million. This would give a range of between 2.2 (additive approach) and 3.8 million (global ratio approach), very much in line with the previously updated estimates of between 2.3 and 3 million (4, 99) and precisely narrowing down the range of 1 to 5.1 million given by Blackwell (10) by 1.2 to 1.3 million on either side.

We therefore propose to replace previous estimates of global fungal species richness with this updated range of 2.2 to 3.8 million. This estimate is likely to be further improved on when reliable statistical approaches to analyze the huge amount of environmental sequence data become available. An interesting approach would be to combine the following techniques: (i) a geographically and ecologically broad sample of environmental sequence data, (ii) alignment-based species recognition methods to properly estimate OTU diversity, (iii) species-based niche modeling to establish macro- and microecological patterns, and (iv) a grid-based interpolation of global species richness, taking into account sampling effort. We predict that such an analysis will result in estimates that might lie well above the revised conservative estimate of 2.2 million species and likely even beyond 3.8 million species. But even if continuing to adopt the previous, now appearing highly conservative number of 1.5 million species, the discovery and formal description of the missing fungi remain a daunting task.

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