Impact of DNA data on fungal and yeast taxonomy

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The species richness and diversity existing in microorganisms are unparalleled on our planet. In recent years, the use of molecular techniques for the detection and identification of microorganisms, especially through DNA sequencing, has revolutionised the way taxonomists look at the systematics of a species, especially when applying multilocus sequence data and concepts such as the genealogical concordance phylogenetic species recognition (GCPSR) concept[1]. Particularly problematic topics arising in the fungi are, for example, naming pleomorphic fungi (that is, fungi for which a teleomorph, meaning sexual form, and one or more anamorphs, meaning asexual forms, are formed), polyphyletic genera (that is, the same genus being presented in several distinct phylogenetic lineages or clades), morphospecies (a species which is differentiated from other species based on its distinctive morphological characters) that in fact represent distinct phylogenetic lineages and how to deal with species that are only known by their DNA sequence(s). Here we provide a brief overview of the effects and implications that the application of molecular techniques such as DNA sequencing are having on the taxonomy of fungi (part 1) and yeasts (part 2), with specific reference to those which can be cultured. Although fungi and yeasts both belong to the kingdom Fungi, sometimes different approaches to the taxonomy of these two 'groups' were followed in the past and, therefore, they are treated individually below. For the purpose of this overview, genera of Oomycetes are excluded.

Part 1: Fungi

It is not clear how many fungal species there are, but previous predictions based on herbarium specimens (1–1.5 M^2), are clearly a vast underestimate (discussed elsewhere[3]). For example, eight unique species were described from a single leaf spot of a eucalypt tree growing in Madagascar[4] and, in another study, 11 novel species (one even representing a novel genus) and six known species were isolated from ten leaf bracts of *Phaenocoma prolifera* ("everlastings") from South Africa[3]. The numbers in the latter two studies are only based on culturable fungi grown from germinating spores, and do not reflect those species that are growing endophytically and/or not actively discharging spores. Once a researcher applies techniques such as 454 pyrosequencing to obtain sequences from all fungal DNA present in a sample, these numbers increase significantly. For example, approximately 30,000 reads of the first internal transcribed spacer region (ITS) of the nuclear ribosomal DNA (nrDNA) operon were recovered from 4 g of forest soil[5]; these reads corresponded to roughly 1000 molecular operational taxonomic units (MOTUs – referring to specimens having identical or within a certain threshold identical sequences; this has been reviewed[6]). However, the downside of such a sequence-based approach is that no living cultures directly linked to the sequence are available to actually study and to conserve the potentially novel species that were sequenced. Additionally, one is dependent on the quality of the obtained sequence and even more on the quality of the reference databases used for sequence-based identification[7]. The use of a notation similar to the "*Candidatus* species" concept proposed and implemented for bacteria as interim taxonomic status for noncultivable organisms[8], was recently proposed by David Hibbett during his keynote lecture at the International Mycological Congress 9 (2010) in Edinburgh to attach a handle to such MOTUs until such a stage where the sequence can be connected again to an organism which can be formally described[9].

If a fungus can be cultured, and it is sporulating, it is possible to describe and name the species based mainly on its macro-

and micromorphology (for example, colony characteristics and spore morphology) and sometimes even its specific host, and to lodge a representative culture, the so-called ex-type culture, in a public culture collection for conservation (a protocol was published last year[10]). Morphological descriptions can also be based on the appearance on host tissue or herbarium specimens, but adding molecular data from such material is more difficult due to the higher risk of obtaining contaminated DNA samples. Naming pleomorphic fungi has always been a controversial process[11], as mycologists are divided about the value of the dual nomenclature system (that is, using one name for the sexual form and a second name for the asexual form of the same fungus, if both forms are produced) in the DNA era (Article 59 of the International Code of Botanical Nomenclature[12]). Recent years saw a gradual move towards a single nomenclature (that is, one name for a specific fungus, irrespective of which form it is producing) for fungal species[13], an approach that has been successfully applied to genera in the *Capnodiales*[14-18] and *Hypocreales* (for example, *Calonectria*[19,20] and *Fusarium*[21,22]). The rationale behind these decisions was to use the oldest generic name for a well-defined molecular phylogenetic group (representing a genus) of fungi, irrespective of whether this represents an anamorph or teleomorph genus. For example, the anamorph genus *Cylindrocladium* (described in 1892) is linked to a single teleomorph genus *Calonectria* (described in 1867) and therefore the authors chose to use the name *Calonectria*[19,20].

Polyphyletic genera are genera that occur at more than one position in a phylogenetic tree, indicating that the same morphological traits have either evolved or were lost more than once during evolution. If only morphology is considered when a species is placed in a genus, this is less of a problem, as the morphology would fit the morphologic description of the genus. However, once a phylogenetic backbone is added, the species can go into any of the "bins" which make up the polyphyletic genus, and therefore the morphologic "Genus X species" might become phylogenetic "Genus X Clade II species". In the past, the genus *Mycosphaerella* was considered to be a single genus with 30-odd associated anamorph genera[16,23]; when data using the 28S nuclear ribosomal RNA gene was added it became clear that "*Mycosphaerella*" in fact represents an assemblage of numerous genera distributed over several families[16,24]. A similar approach has also been followed in, for example, *Botryosphaeria* and its anamorph lineages[14,15]. When attempting to delineate polyphyletic genera in such a way as to provide long-term taxonomic stability of names to the end-users – frequently by applying novel generic names to the individual phylogenetic clades based on characters unique to each clade – one has to retain one clade as definitive of the original genus. This clade is normally characterised by it containing the type species originally designated when the genus was established. This is relatively easy to do if authentic ex-type cultures (that is, cultures derived from the type specimen) of the species are available. However, if ex-type cultures or holotype (that is, a dried inactive specimen presenting all the diagnostic morphological characters of the species) material, which are used to pin down the name of the species are not available because the material is lost or the specimen is depauperate, or in the case of cultures even dead, it becomes difficult to confirm which clade to use as representative of the genus as the molecular data from the type species can not be added. For example, the type material of *Mycosphaerella punctiformis* (type species of the genus *Mycosphaerella*) was never designated and, therefore, no material was available that can be used to assign this species unequivocally to a phylogenetic lineage. Through the processes of lectotypification (that is, the process of defining a type specimen if the holotype specimen was not originally designated) and epitypification (that is, the process of designating a specimen or illustration which can serve as an interpretative type of a species in the absence of other reliable material) it was possible to assign type status to a culture derived from strains of the species recollected from its host[25].

For many genera and species, no material/cultures with type status are available, making it difficult to assign the correct name to a phylogenetic lineage, especially if a morphospecies is shown to be a species complex based on molecular methods. For example, *Cercospora apii* and *Cercospora beticola*, long considered to be synonyms[26], were distinguished using a polyphasic approach incorporating multilocus sequence data, amplified fragment length polymorphism (AFLP) fingerprinting and growth rate/optimal growth temperature requirements as criteria[27]. The authors then designated epitype specimens and strains with which to fix the names to specific cultures representative of each species. In other studies, for example, the *Fusarium graminearum* species complex, responsible for Fusarium head blight on wheat and barley, was split into nine phylogenetic species based on multilocus sequence data[28] and the *Neofusicoccum parvum/N. ribis* complex was shown to contain at least three more cryptic species[29]. One of the biggest implications arising from morphospecies representing distinct phylogenetic species is the question of how accurate are lists based solely on morphology to represent the local biodiversity in a country[30]? This is particularly so if these lists are used for quarantine purposes to identify which species are already present in a country and which should be prohibited. To this regard, a DNA barcoding project called QBOL (Quarantine Barcode of Life) was funded by the European Union to start generating a database (QBANK[31]) containing DNA sequences of vouchered organisms of quarantine importance to Europe, as well as close relatives of these species.

Part 2: Yeasts

Often when the term "yeast" is used, it is with a mental picture of *Saccharomyces cerevisiae* in mind and this is why *S. cerevisiae* sensu stricto are referred to the "true yeasts" and are classified in the order *Saccharomycetales*; but, yeasts in the broad sense are not monophyletic. Before the molecular era of yeast taxonomy, yeast taxonomy was based on morphology, mode

of sexual reproduction, physiology and biochemical activities, placing taxa within their respective taxonomic groups. However, molecular comparisons have shown that many of the earlier identifications based on phenotypic tests are incorrect. The molecular era started with the determination of the mol% G+C relations of nuclear DNA[32], followed by the DNA reassociation technique[33]. The latter method had a big impact on yeast taxonomy, but it is time-consuming and the cut-off values for con-specificity are quite vague (70–80%). During recent years it became clear that important morphological characters such as the shape of sexual spores (for example, *Kodamaea ohmeri*[34] and *Metschnikowia lachancei*[35]) as well as specific physiological characters (for example, fermentation assays screen only for CO_2 production, which might result in a false-negative if the amount of CO_2 produced is below the detection limit[36,37]) that were used extensively in the past for species delimitation have limited value in this regard. This information is still used in species descriptions and is valuable for ecology and industry. Molecular comparisons offer an unprecedented opportunity to re-evaluate current taxonomic schemes from the perspective of quantitative genetic differences; already it is evident that yeasts are not only restricted to the *Saccharomycetales* but have members not exclusively belonging to the ascomycetous fungi but also the basidiomycetous fungi. Nuclear ribosomal DNA sequences, especially the D1/D2 domains of the 26S nrDNA and to a slightly lesser extent the internal transcribed spacers of the nrDNA operon, have been more commonly used for yeast taxonomy and phylogenetics, resulting in a huge sequence database for species identification. In contrast, other loci such as mitochondrial DNA and protein coding genes are only now becoming more popular as alternative or additional molecular markers for species identification and delimitating species boundaries. Based on the molecular data obtained thus far, several changes have been introduced in the classification of yeasts; however, substantial restructuring of current taxonomic schemes with the consequence of numerous nomenclatural changes await ongoing studies using a multilocus sequencing approach and molecular characterisation of previously unsequenced species.

Several past studies[38-41] combined represent a huge first attempt to show that sequence data, in particular the D1/D2 region of the 26S nrRNA gene, can be used effectively as a marker for a specific yeast species. A species was then defined as a group of strains generally having less than 1% nucleotide differences in the D1/D2 region[39]. Data obtained from D1/D2 sequences were found to correlate fairly well with the DNA reassociation values[38-42]. In a multigene sequence study, using five gene regions, 75 well-known species that belong to the *Saccharomyces* complex were placed into 14 phylogenetic clades, many of which were separated from their originally described taxonomic group[43]. This study was the stepping-stone of many follow-up studies in which numerous known species were reassigned to different genera and groups as well as the description of a substantial number of new combinations and new species using different gene regions. These studies include the reassignment of *Stephanoascus ciferrii* and *S. farinosus* to *Trichomonascus* and *S. smithiae*, together with linking several *Candida* species, to the newly described genus *Sugiyamaella*[44]. In the same year, 11 new *Sugiyamaella* species were described[45], with both studies using the nearly complete 26S nrDNA (LSU), mitochondrial small subunit (mtSSU) and cytochrome c oxidase subunit 2 (COXII) regions. Using LSU, 16S nrDNA (SSU) and translation elongation factor 1-alpha gene sequences, numerous *Pichia*, *Issatchenkia* and *Williopsis* species were assigned to the newly defined genera *Barnettozyma*, *Lindnera* and *Wickerhamomyces*[46]. The genus *Debaryomyces* and related *Pichia* species were shown to be polyphyletic[47], resulting in 23 new combinations within the newly described genera *Babjeviella*, *Meyerozyma*, *Millerozyma*, *Priceomyces*, *Scheffersomyces* and the reinstated and amended *Schwanniomyces*. As the D1/D2 and ITS region were not successful in resolving closely related species in the *Debaryomyces hansenii* complex the actin gene was found to be a valuable gene region for species delimitation within this complex[48]. The actin gene region was also shown to be a good marker for species delimitation of various closely related medical taxa[42]. The taxonomy of medically important yeasts was also studied using the multigene sequencing approach and revealed that the three groups described in *Candida metapsilosis* were actually three separate species, *C. metapsilosis*, *C. parapsilosis* and *C. orthopsilosis*[49].

In contrast to the ascomycetous yeasts, the delimitation of a basidiomycetous species based on sequence analysis, still needs considerable attention as the anamorphic genera *Cryptococcus*, *Rhodotorula* and *Sporobolomyces* are present in more than one phylogenetic group[41,50]. Limited studies have thus far used a multigene sequencing approach in order to refine the phylogenetic relationship among the species. Sequence analyses of the D1/D2 and/or ITS regions have been used for the classification of a large number of the basidiomycetous yeasts species[41], but the phylogenetic tree structures are not fully resolved and several strongly supported clades contain polyphyletic or poorly resolved genera. This resulted in insufficient evidence for making adequate taxonomic decisions, particularly at the generic level, demonstrating that other genetic regions must be examined to distinguish these taxa. The transfer of the five species of the genus *Trichosporonoides* to the genus *Moniliella* was proposed only on the basis of D1/D2 sequencing and phenotypic characteristics[51] whereas *Trichosporon pullulans* was reassigned to *Guehomyces pullulans* and *Hyalodendron lignicola* to *Trichosporon lignicola* on the basis of divergence in the D1/D2 and ITS regions[52]. Extensive molecular studies are done on the *Cryptococcus neoformans – C. gattii* complex as these species can cause meningoencephalitis in immunocompromised as well as in immunocompetent patients. The genetic diversity among strains from these two closely related species was found to be significant as more than 10 different genotypic groups have been distinguished[53]. It is evident from these data that many of these

lineages will probably be considered as different taxa or groups in the near future. However, according to recent studies on the molecular determination of a number of these genetically diverse subgroups, it seems that the species delimitation within these groups is not so easy as different hybrids between the two *Cryptococcus* species has also been found[54]. A large basidiomycete yeast barcoding project between Chinese and Dutch collaborators is currently in progress, using a multigene sequencing approach in order to get a clearer view on the taxonomy of this important group of yeast genera. This effort will increase the reliability of the taxonomy of the basidiomycete yeasts and will make a huge contribution to yeast taxonomy in general.

Sequence typing has resulted in a doubling of the number of known yeast species in the past 13 years[55,56]. In 1998, 41 and 15 ascomycetous teleomorphic and anamorphic genera were listed, respectively, and 20 teleomorphic and 21 anamorphic basidiomycetous genera were listed, whereas currently, 73 teleomorphic and 14 anamorphic ascomycetous genera and 34 teleomorphic and 28 anamorphic basidiomycetous genera are recognised. From the first to the current edition of *The yeasts, a taxonomic study*, the number of species described has increased from 164 in 1952, 349 in 1970, 500 in 1984, 700 in 1998 to 1500 in 2011[55-57].

As the D1/D2 and in the some cases also the ITS regions have been sequenced for many yeast species, these regions are currently the preferred target for sequence-based identification of yeasts and as the basis of taxonomic decisions. However, this will change rapidly as there is a drastic increase in the number of sequences of additional gene regions for several taxonomic groups and this will lead to an even more rapid increase in the number of yeast genera and species. If one extrapolates the current growth rate in number of species, it can be predicted that within the next 13 years, the number of yeast species would reach 3000. However, when new DNA sequencing platforms incorporating all environmental DNA are considered, this number will be proven to be a vast underestimate.

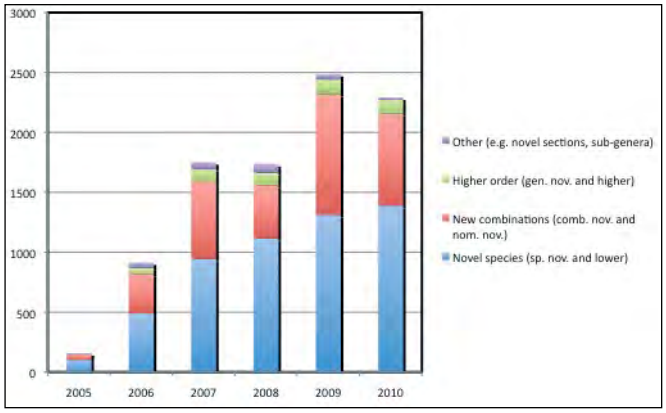

Figure 1. Number of taxonomic novelties registered in MycoBank since the launch of the database in 2004. The numbers of novel species and combinations are especially striking. Particularly the new combinations can be ascribed to the refinement of systematics due to the inclusion of molecular data in the taxomic treatment of species, resulting in known species names being combined into another genus.

Conclusions

The taxonomy of yeasts and filamentous fungi are presently in an exciting, but challenging era (recently reviewed[58]). The use of molecular tools are expediting species discovery; however, naming these species is hampered because of the thousands of names described in the last 200-odd years. Fortunately, initiatives such as MycoBank (Figure 1)[59,60], Index Fungorum[61] and the *Dictionary of Fungi*[62] provide some guidance on described names and taxonomic changes. However, for many of these species names, insufficient descriptions were provided and, even if it would have been possible at that time, no cultures or frequently even herbarium specimens were kept. Therefore these species will have to be recollected from their original host and locality to add molecular data to confirm their phylogenetic relationships. What will/should happen to these 'old' names remains open to speculation; at the moment mycologists have learned from the past and are designating type materials for novel species as far as possible, and placing these in herbaria, culture collections, DNA banks, nomenclature and DNA sequence databanks, so that these are available for future generations, and can become fully incorporated into the Encyclopedia of Life[63]. In the future, it is quite possible that the use of whole genomes for phylogenetic comparisons ("phylogenomics", published examples exist[64]) might again redefine our understanding of the processes underlying the evolution of species and the resulting fungal tree of life.

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