'Species concept' in microbial taxonomy and systematics

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Species is the basic unit of identification of a large diversity of organisms. The concept of species is old and has undergone several changes depending on the kind of organism(s). Earlier concepts were formulated considering mostly macroorganisms (plants or animals). In 21st century the microbial species concept took shape which changed from morphological, ecological, biological and phylogenetic to more agreeable polyphasic species concept. Polyphasic species concept involves a combination of characters such as morphology, physiology, biochemical markers and sequencing of various regions of the DNA known as molecular markers. There are also specific criteria such as DNA–DNA hybridization for complex genera of Bacteria and Archaea, extrolite profiling for complex genera of fungi, etc. Although species concept and taxonomy of Bacteria are more systematic than fungi, taxonomy in two domains (bacteria, archaea) and fungi of eukarya domain is undergoing changes. The time is not far when genome sequencing will be considered as another criterion for delineating species. This article also discusses the role of various techniques (MALDI, FAME, etc.) in species identification. The concept of Candidatus species and species annotation for metagenomic studies is also discussed. We briefly describe the criteria used to delineate species in various microbes and the need for different species concepts based on the kind of organism.

Keywords: Archaea, Bacteria, fungi, species concept, taxonomy and systematics.

'SPECIES CONCEPT' has been in use ever since the realization that classification of organisms is important in biology. Species is considered as a fundamental unit of comparison in virtually all subfields of biology, from anatomy to behaviour, development, ecology, evolution, genetics, molecular biology, paleontology, physiology and systematics[1,2]. It consists of population(s) of organisms with similar features and monophyletic origin, and often defined as a group of organisms capable of interbreeding and producing fertile offspring[3]. Thus, reproduction was regarded as one of the important species differentiation factor which still holds good for many organisms, but not so in the case of microbes. As organisms reproduce and genes replicate, changes occur in populations thus accomplishing speciation (i.e. species change). Harrington and Rizzo[4] had emphasized the need for a simple species concept useful for plant pathologists, medical practitioners or diagnostic workers. A clear demarcation between species of a genus helps in various applications such as quarantine regulations, pharmaceutical industry, patent applications, biofertilizer manufacturers, etc. Thus there is the need for a species concept applicable to bacteria, archaea and fungi (non-lichen).

Most of the earlier reviews on species concepts were concerned with plants and animals[5] which are relatively easy to classify based on morphological features. In contrast, the microbes have a simple morphology and thus warrant polyphasic approaches for their classification and delineation at the species level. This article discusses developments in microbial taxonomy, former and current systems of classification, species concepts and criteria used to achieve them.

Microbial species concepts and criteria

Species concepts in true fungi (Eumycota)

Fungi occupy almost all niches of the earth and form a variety of morphological structures such as macrofungi (Agaricales, *Peziza* cups, jelly fungi, bracket fungi, Corticiaceae members, etc.) and microscopic fungi (forming ascomata, pycnidia, sporangium, zygospores, etc.). Furthermore, some fungi have two stages in their life cycle (sexual or teleomorph and asexual or anamorph stages), while others show only asexual or sexual stage. Thus, a variety of genetic systems, life-history patterns and intraspecific interactions in fungi give rise to new

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variants and maintain unique adaptations[6]. A lot of ambiguity in fungal species concept has been observed due to diversity in morphology and physiological specialization (rust and smut causing fungi), so much so that a plant pathogenic fungus found on a new host was considered a novel organism. The new trend of using molecular data has helped resolve some of these taxonomic issues related to morphology and physiology of fungi and has also emphasized the importance of the use of standard growth conditions (pH, temperature, medium, incubation period, etc.) for describing a novel fungus. Earlier, species delimitation in fungi was primarily by morphology, physiology, host specificity and interbreeding. Shin *et al.*[7] separated dermatophytic fungal species on the basis of mating experiments. Gibas *et al.*[8] described a cryptic novel species (*Arachnomyces glareosus* anamorph *Onychocola glareosus*) based on the mating experiment and ITS data distinguishing it from *Onychocola sclerotia*. It is not commonly used as it is difficult to infer and conclude from the observations[9]. However, it can be used for strain differentiation or to ascertain the relationship of some anamorphic deuteromycetes fungi with their teleomorphic stage.

Sequencing of DNA regions and subsequent cladistics/phylogenetic studies in taxonomy has changed the concept. In fungi, it has helped in the identification of only asexual and non-sporulating forms, especially animal or human pathogens (usually non-sporulating inside the host). According to the current species concept followed by the International Commission on the Taxonomy of Fungi (ICTF) or International Code of Botanical Nomenclature (ICBN), a species is delineated by phenotypic characters, ecological niche, morphology, physiology, molecular markers and in some genera, extrolite profiling. Some of the criteria are discussed below.

Morphological characters: These have influenced species delimitation in fungal taxonomy. For asexual forms, quantitative morphological features such as colony (configuration, elevation, margin), conidia and conidiophore (colour, shape, size, etc.), type of conidia, conidiogenesis (blastic and thallic), chlamydospores, etc. have been considered for taxonomic purpose. Whereas in fungi with sexual stages, a variety of morphological details have influenced fungal taxonomy. In Ascomycota, ascus and ascospore dimensions help in species delineation. *Corynascus verrucosus* and *Corynascus sepedonium* are separated based on the germ pore on ascospore and surface of ascomata[10]. Similarly, in Basidiomycota the shape, size of fruiting body, colour of spore, reaction to Melzher's reagent, details of lamellae (in gills), mycelial structure of stipe and cap, etc. form important characters of species identification. In a study, Sharma *et al.*[11] found that as per the ITS sequence data and RFLP of ITS region, strains of *Microsporum gypseum* which formed appendages were similar to other strains of the same species which did not form appendages. It resulted in synonymizing *M. appendiculatum* (proposed only on morphological criteria) to *M. gypseum*. In wood-inhabiting Corticiaceae members, many are still identified based only on morphological characters[12]. Improvement in light microscopy (such as phase contrast, Nomarski differential interference contrast microscopy), ultra microtomy, SEM, TEM, differential staining techniques, etc. have helped in refining the criteria for species identification.

Phenotypic characters for obligate fungal parasites: In the present acceptable system of fungal species identification for phenotypic characters, a herbarium specimen, which may be a leaf or stem infected with fungus, dried culture plate, etc. has to be deposited in a herbarium with an on-line database so that the material is available on request to scientists. It should show most if not all features/characters of the fungus. The author has to designate it as 'holotype' and the culture derived from it is called as 'isotype' or 'ex culture type'[13] deposited in internationally recognized culture collection(s).

Ecological adaptation and physiological characters: Ecological niche is an important factor for speciation and species description (commonly used for plant pathogenic fungi and mushrooms). The ecological niche data help delineate strains, if not species, within a genus. With time species change their niche and adapt to new habitats. A species which is usually considered non-pathogenic and inhabiting cellulolytic wastes can become pathogenic. A strain of *Trichoderma harzianum* (other strains of which are used as biofertilizer in crops) is reported to cause infection in an immune-compromised patient[14]. Adaptation to new niches, different temperatures, pH, etc. also play an important role in speciation. Hence, physiological characters are considered to be an essential factor for species delineation. In a study on *Chaetomium*, Li *et al.*[15] used minimum and maximum temperature tolerance to categorize strains. Similar factors are used in the characterization of fungi such as *Penicillium*, *Aspergillus*, *Fusarium*, etc.[16]. In *Penicillium* and *Aspergillus* production of acid on creatine sucrose agar medium (CREA) is used as a semi-selective medium for classification[17].

Secondary metabolite and other biochemical markers: Chemotaxonomy in fungi is limited to the use of fatty acids, proteins, carbohydrates as a criteria for identification. Secondary metabolites as taxonomic criteria are not used much in fungi as they are not consistent and include a combination of many metabolites. It is still considered as an additional character, but not an essential one. Till now, fungal chemotaxonomy based on secondary metabolites has been successfully used in many large ascomycete genera such as *Alternaria*, *Aspergillus*, *Fusarium*, *Hypoxylon*, *Penicillium*, *Stachybotrys*, *Xylaria* and in few Basidiomycete genera, but not in Zygomycota and

Chytridiomycota[18]. Secondary metabolites have been an important character for the Xylariaceae family[19]. In this family, apart from morphological characters, the pigment production by stromata of *Daldinia* and *Hypoxylon* is taken as the criterion for species separation. These are considered to be stable and can be easily detected. Stadler *et al.*[20], while describing polyphasic taxonomy of *Daldinia*, have studied secondary metabolite profiles of several species which were characteristic of that species. Other biochemical markers such as the Ehrlich reaction are used for the classification of *Penicillium* subgenus *Penicillium*, *Aspergillus* section *fumigati* and *Aspergillus* section *nigri*[16]. Extrolite profile of species in genera *Aspergillus* is recommended by ICBN for species delineation[17,21].

Molecular/phylogenetic data: Molecular markers (RAPD, RFLP, DNA sequencing, etc.) have revolutionized the taxonomy of fungi. A few years ago Hibbett and his group had proposed a new fungal classification based on DNA sequence data, which is widely accepted[22,23]. Sequencing of nuclear and mitochondrial ribosomal RNA gene (18S rRNA small subunit (18S-SSU), 28S ribosomal RNA large subunit (28S-LSU), internal transcribed spacer (ITS), intergenic spacer (IGS1) region, mtSSU and mtLSU), as well as protein-coding genes, such as RNA polymerases II (*rpb1* and *rpb2*), β-tubulin (*β-Tub*), calmodulin (*cal*), γ-actin (*act*), ATP synthase (*atp6*) and translational elongation factor 1α (*EF-1α*), etc. has been used for identification. A summary of different regions used for different genera is given in Table 1. The ITS region has been recognized as a marker for barcoding of fungi after screening six DNA regions, including protein coding genes[24]. The ITS region of fungi consists of ITS1-5.8S-ITS2. The 5.8S region is a highly conserved region in all groups of fungi and does not help much in taxonomy of fungi. ITS1 and ITS2 regions show variation between species with different percentages of variation. The ITS1 region of *Arthrinium* is more variable than ITS2, 72% and 39% respectively[25]. However, for yeast it was found that LSU gives better resolution than ITS region in species identification and hence data from both regions are required. Certain fungi are phylogenetically distinguished by only ITS sequence data like *Arthrinium*, *Pseudofusicoccum*, etc.[25,26], whereas others are not distinguished like *Corynascus*, *Scedosporium*, etc.[10,27], and multi locus sequence typing (MLST) is adopted for the latter. For example, MLST of *Cryptococcus neoformans/C. gattii* species complex is resolved using capsule polysaccharide (*cap59*), glyceraldehyde 3-phosphate dehydrogenase (*gpd1*), laccase (*lac1*), phospholipase B1 (*plb1*), manganese superoxide dismutase (*sod1*), orotidine monophosphate pyrophosphorylase (*ura5*) genes and the IGS1 region[28]. MLST has thus become a handy tool to delineate *Scedosporium apiospermum*, *Pseudallescheria boydii* and *Chaetomium jatrophiae*[29,30]. In all these studies the number of genes analysed ranged from 1 to 5 (ITS, SSU, LSU, *β-Tub* and *EF-1α* are commonly used). MLST sequencing has helped separate several cryptic species, viz. *Fusarium oxysporum*, *Candida albicans*, *Trichoderma viride*, *Aspergillus niger* and *Histoplasma capsulatum*. In resupinate Homobasidiomycetes (*Corticiaceae sensu lato* and others), phylogenetic studies done using rRNA gene sequences have restricted the bigger group to a small Corticiales[31]. Thus MLST (which involves 7–8 genes) has a great potential in species separation in microbes[32]. Such rRNA gene sequence data help to correctly identify a white-coloured mushroom and differentiate it from an albino of a known coloured species. Single-copy protein-coding genes such as *RPB1* and *RPB2* are promising for yielding well-resolved and highly supported phylogenies[33]. However, other protein-coding genes such as *β-Tub*, which are present in multiple copies in the fungal genome may be phylogenetically misleading[34]. Generally it is considered that slow-evolving loci are more suitable for reconstruction of deep phylogenetic relationships, while loci with high rates of evolution are better for the reconstruction of more recent evolutionary events. Moreover, genes which undergo mutation easily or get affected by changes in the environment should not be used for phylogenetic studies. Mayden[35] and Taylor *et al.*[36] consider it as a genealogical concordance phylogenetic species concept which relies on the concordance of more than one gene genealogy. Due to the resolution power of molecular markers and repeatability, this technique has become a primary tool for characterizing strains.

Species concept in Bacteria and Archaea

Recent studies using next-generation sequencing approaches have indicated that the gut of an insect harbours 1479 operational taxonomic units (OTU) (in wood-feeding huhu beetle larvae (*Prionoplus reticularis*))[37], the termite gut 4500 bacterial species[38], plant endophyte harbours 45–689 strains[39] and human gut harbours 500–1000 bacterial species with an estimated 10^{14} bacterial cells[40,41]. If this is the estimate, the number of estimated bacteria on the earth would be mind-boggling.

Earlier Bacteria were identified based on morphology, physiological traits and chemotaxonomic properties. Archaebacteria were discovered in 1977 and proposed as a separate domain based on their phylogenetic diversion by Carl Woese. Similar to bacterial taxonomy, 16S rRNA gene sequences are used to ascertain the taxonomy of Archaea at higher taxonomic levels. A characteristic peptidoglycan containing N-acetyl talosuronic acid in the place of N-acetyl muramic acid is observed in certain Gram-positive members of the Archaea. A variety of side chains has been recorded. The range of polar lipids known to occur in members of the Archaea is currently restricted to phospholipids, aminophospholipids, glycolipids and phosphoglycolipids. Members of the Bacteria have a diverse range of hydrophobic side chains in their

Table 1. Some examples of variation in species criteria used to delineate species in different genera of microbes

Genera	Morphology and physiology	Molecular	Biochemical
Taxonomic criterion for species delineation			
Fungi			
Arthrinium	Conidia and conidiophores shape, dimensions, temperature range, pH range	ITS region, LSU, *EF1-α* and *β*-tubulin gene	–
Alternaria	Conidia, conidial septation, beak branching, conidiophore dimensions, colony morphology, temperature range, pH range	ITS region, *gpd*, *Alt a1*, calmodulin gene	Extrolite profile
Aspergillus	Conidial dimensions, columella, vesicles, sclerotia, colony morphology on prescribed media, temperature range, pH range	ITS region, LSU, *β*-tubulin, calmodulin, *benA*, *caM* and *rpb2* gene (depending on section)	Extrolite profile
Botryosphaeria	Conidial dimensions, colour, conidiogenous cells, pycnidia, Temperature range, pH range	ITS region, SSU, LSU, *β*-tubulin and *EF1-α* gene	–
Chaetomium	Ascomata, ascospores and conidia dimensions, lateral and terminal hairs, pycnidia, temperature range, pH range	ITS region, LSU, *β*-tubulin and *EF1-α* gene	–
Colletotrichum	Conidial dimensions, colony morphology, appressoria, temperature range, pH range	ITS region, *β*-tubulin, *gpd* and histone *H3* gene	–
Fusarium	Conidia and chlamydospore dimensions, sexual compatibility, colony morphology, temperature range, pH range	*β*-tubulin, *EF1-α*, histone *H3* and calmodulin gene	Extrolite profile
Penicillium	Conidia dimensions, phialides shape and size, growth rate, colony morphology, temperature range, pH range	ITS region, LSU, *β*-tubulin, calmodulin, *rpb2*, *EF1-α*, *CO1* and *benA* gene (depending on complex)	Extrolite profile
Phoma	Chlamydospores, conidia shape and size, pycnidia, temperature range, pH range	ITS region, SSU, LSU, actin and *β*-tubulin gene (depending on section)	–
Pseudofusicoccum	Conidia, conidiogenous cells, conidiomata, temperature range, pH range	ITS region	–
Trichoderma	Conidia shape and size, phialides shape and size, chalmydospore, growth rate, colony morphology, odour, temperature range, pH range	ITS region, LSU, *EF1-α* and *rpb2* gene	–
Daldinia	Ascospore dimensions, germ slit morphology, conidiophore and conidia dimensions, ascospore ornamentation, stromatal habit, morphology of ostioles and perithecial outline, temperature range, pH range	ITS region, LSU, *β*-tubulin and actin gene	Extrolite profile (stromatal pigments)
Scedosporium	Conidia, conidiogenous cells, temperature, pH	ITS region	–
Microsporum	Conidia dimensions, ascomatal appendages, temperature range, pH range	ITS region	–
Trichophyton	Conidia dimensions, ascomatal appendages, temperature range, pH range	ITS region	–
Auxarthronopsis	Peridial appendages, ascospore ornamentation, temperature range, pH range	ITS region, LSU and SSU gene	–
Auxarthron	Peridial appendages, ascospore ornamentation, temperature range, pH range	ITS region, LSU and SSU gene	–
Leucoagaricus	Lamellae, cheilocystidia, pileus covering, pileus shape and colour	ITS region, *EF1-α* gene	–
Bacteria			
Myceligenerans	Mycelia position, Gram-staining, pigment production, temperature range, pH range, NaCl tolerance	16S rRNA, DNA–DNA hybridization, G + C content	Enzyme test, antibiotic sensitivity test, substrate utilization
Rhodopseudomon	Cell shape, size, type of budding, colour of culture, pH range, temperature range, oxygen requirement, light requirement, growth factor requirement	16S rRNA, DNA–DNA hybridization, G + C content	Carotenoid composition, whole-cell fatty acid analysis, whole-cell absorption spectra, polar lipid profile
Siccibacter, Franconibacter	Cell shape, size, motility, pH range, temperature range	16S rRNA, *α*-glucosidase, *rpoB*, *zpx* (zinc metalloprotease), and *cgcA*, (diguanylate cyclase) genes	Acid production from carbon sources, Voges–Proskauer, methyl red, production of enzymes
Archaea			
Nitrososphaera	Cell appendages, ultra cell structure with TEM, S-layer and its symmetry, temperature range, effect of pyruvate and ammonium	16S rRNA, *amoA* and *amoB* genes	Intact polar lipids and glycerol dibiphytanyl glycerol tetraether (GDGT), C and N compounds on growth

lipids, whereas members of the Archaea have only isoprenoid-based side chains. Till now, isoprenoid-based ether-linked lipids and isoprenoid ether-linked side chains have been detected only in Archaea[42].

Woese and Fox[43] were the first to propose 16S rRNA gene sequence as a reliable phylogenetic marker for various bacterial species and it became mandatory to incorporate full length 16S rRNA gene sequence (~1500 bp) in bacterial polyphasic taxonomy as recommended by the International Union of Prokaryotic Nomenclature (IUPN) to characterize species in prokaryotes[44]. This polyphasic approach not only standardized but also stabilized bacterial taxonomy. The concepts of speciation applicable to other eukaryotic organisms such as sympatric species (speciation due to reproductive isolation) and allopatric species (speciation due to geographical isolation) are difficult to relate to bacteria. According to Staley[45], Bacteria which share an evolutionary history and clade together in the phylogenetic tree are categorized as one species. Such species also need to exhibit similarities with respect to various phenotypic and genotypic characters, including greater than 70% similarity at DNA–DNA hybridization level and greater than 97.5% similarity at the 16S rRNA gene sequence level. Thus polyphasic taxonomy (morphology, physiology, chemotaxonomy), including G + C content of DNA, DNA–DNA hybridization[46–48] and 16S rRNA gene sequence is the gold standard for species assignment in Bacteria and Archaea. Although 16S rRNA gene is considered to be one of the important characters in differentiation, in certain cases the above criteria may not be sufficient and warrants the use of MLST to differentiate Bacteria at species level. The genes normally sequenced for MLST in bacterial species include carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*), glucose-6-phosphate isomerase (*glp*), DNA gyrase, subunit B (*gyrB*), malate-lactate dehydrogenase (*mdh*), methionyl-tRNA synthetase (*metG*), phosphoribosylaminoimidazole synthetase (*purM*), threonine dehyrogenase (*dtdS*), diaminopimelate decarboxylase (*lysA*), transhydrogenase alpha subunit (*pntA*), dihydroorotase (*pyrC*) and tryptophanase (*tnaA*)[49,50]. Certain environmental factors such as light intensity, temperature, etc. are considered as the criteria for strain differentiation in Bacteria. According to Rosselló-Mora and Amann[47], a species is 'a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity in many independent characteristics, and is diagnosable by a discriminative phenotypic property'. Rosselló-Mora and Amann[47] and Staley[45] have reviewed the prokaryotic species concept. The ability of bacteria to exchange genes within and between species, genera and/or and phyla plays an important role in bacterial species development. The frequency of horizontal gene transfer (HGT) is high in Bacteria compared to other groups of organisms. Apart from this, adaptation to the niche plays a key role, contributing to speciation. The typological species concept is also applicable and the phenotypic and genotypic attributes of a group of organisms should be similar to the designated type. The terms 'ecotypes' and 'geotypes' have been coined to demarcate ecological adaption of different strains of a species (either different vertical layers or temperatures in the sea or other niches). This helps in strain-level differentiation, like two strains of the same species found at different depths of the sea or with different temperature tolerance.

Recent changes to microbial species concept and taxonomy

Since fungal identification began, there were two names (one for teleomorph and one for anamorph) for pleomorphic fungi (Figure 1). Some species were found to reproduce asexually until their sexual stage was discovered. Till the DNA-based sequencing technology became popular in fungal identification (in the 1980s), morphological dimorphism led to two names for one fungus. The DNA sequence data (based on ITS similarity) helped resolve the situation by confirming that the two strains, one forming asexually and another forming sexually may be one. This has made the work of mycologist more difficult as they now have to choose one name for pleomorphic strains like anamorphic *Penicillium* with teleomorphs *Talaromyces* and *Eupenicillium*. During the 18th International Botanical Congress held in Melbourne in July 2011, the dual naming system was changed to a single-name system in Melbourne Code 2011. The principle of priority determines the correct name irrespective of whether it was originally published for teleomorphs or anamorphs. However, the problem remains because many anamorphic fungi are associated with more than one teleomorph genera belonging to different families (*Acremonium*, *Verticillium*, *Cladosporium*, etc.). Moreover, a valid publication does not compulsorily require a Latin name, instead an optional English diagnosis can be done. Nowadays electronic on-line publication is considered valid (earlier a hard print was essential). Deposition of fungal name to accepted repositories (Fungal names, MycoBank, Index Fungorum) is desired for valid publication[51].

Microbial databases used in microbial taxonomy

Microbial databases have become an important tool for accomplishing species identification. The database of National Centre for Biotechnology Information (NCBI), UNITE (http://unite.ut.ee/index.php), Central Bureau Schimmelcultures (CBS) and databases of fungi such as *Fusarium*, *Saccharomycetes*, *Aspergillus* and *Penicillium* are enriched day by day for sequences of different regions.

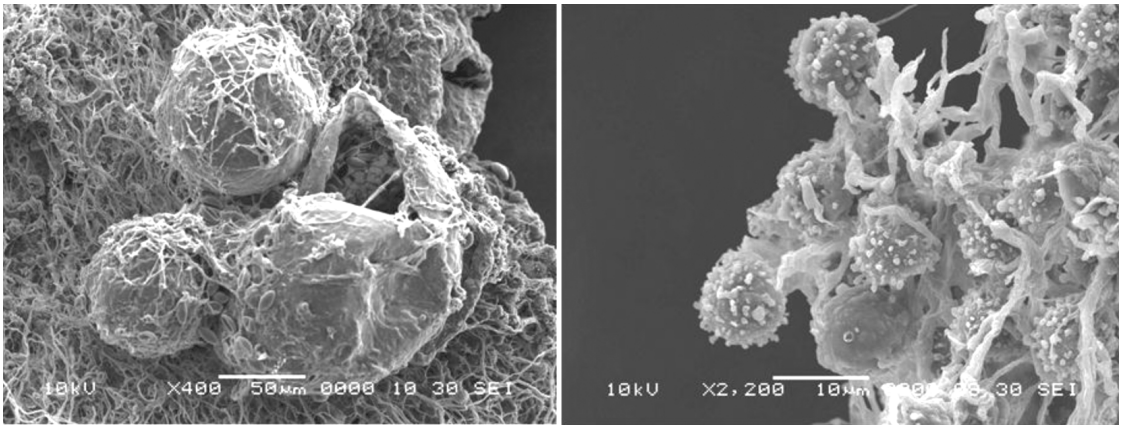

Figure 1. One fungus two names. *Corynascus verrucosus* is a pleomorphic fungus which reproduces sexually (teleomorphic stage) by forming ascospores within ascomata (left), and asexual form *Myceliophthora* sp. (anamorphic stage) reproduces by forming conidia (right). (Image courtesy of Rohit Sharma (left) and Sharma *et al.*[10] (right)).

However, in fungi there are several unauthenticated or wrongly annotated fungal sequences which are problematic for fungal taxonomists. Hence there is a need to sequence all type strains of fungi and update the ITS database[52] because sometimes it gives closest similarity with non-authentic strains or even with unpublished strains of fungi. An environmental sequence with no match in GenBank may be described as an unsequenced species (as many earlier species still remain unsequenced). Bates *et al.*[53] have emphasized the problem of misidentified sequences (including environmental sequences) submitted to public databases. Even in tree-based approaches it may mislead in the identification. Moreover, there is no point in accelerating the research on environmental sequence if we do not have authentic sequences to assign them. Schoch *et al.*[54] have reannotated and/or verified the sequences obtained from either type specimen or ex-type culture and deposited at NCBI as RefSeq Targeted Loci (RTL). MycoBank database registers all new names (species, genus, family, etc.) with a brief description and provides a registration number. A single database consisting of sequences derived from type specimen and/or culture of various fungi is necessary for authentic identification and phylogenetic studies (similar to EzTaxon for Bacteria). Broadly, prokaryotic taxonomy seems to be more systematic than fungal taxonomy. Just as valid novel Bacteria and Archaea names are published in the *International Journal of Systematic and Evolutionary Microbiology* (*IJSEM*), there is a need for publication of a list of valid published names of fungi by ICTF and/or ICBN. In the long run, a unitary nomenclature system for pleomorphic fungi, along with other changes, will promote effective communication[55]. Some other important fungal databases and bioinformatics tools useful for a fungal taxonomist are Index Fungorum, Q-Bank Fungi Database, AFTOL (All Fungal Tree Of Life), SGD (*Saccharomyces* Genome Database), TrichoKEY, Fusarium-ID, The *Aspergillus* website, Mycocosm (1000 Fungal Genome Project), CBS Databases of various fungi, ISTH (International Commission on *Trichoderma* and *Hypocrea*) and Silva (for high quality rRNA databases).

Current trends in microbial species concept and taxonomy

Candidatus species in microbes: *Candidatus* status can be given to prokaryotes which are difficult to cultivate, but relatedness with the closest neighbour can be determined and authenticity verified. The International Committee on Systematic Bacteriology accepts and implements concept of *Candidatus* species in the Bacteria according to the suggestion by Murray and Schleifer[56]. Descriptions from such a category not only expect phylogenetic data but also information on morphological and ecophysiological features and how they can be retrieved *in situ*, together with the natural habitat of the organism. Since *Candidatus* is a status and not definitive rank, efforts are always needed to isolate and characterize this putative taxon for definitive classification[47].

Similar to bacterial and archaeal taxonomy, fungal taxonomy should also consider *Candidatus* species for those strains which are known by more than one character, i.e. some morphological character apart from the sequence. Hibbett *et al.*[57] proposed a parallel taxonomy (or integrated into the present system) for uncultured fungi. They suggested either adoption of *Candidatus* species classification (just as prokaryote) or naming molecular operational taxonomic unit (MOTU) under the ICBN (by extending the definition of type to include environmental samples or sequence illustrations) for uncultured environmental sequences of fungi. Kirk[58] described a new genus of rumen chytrid *Piromyces cryptodigmaticus*, based on sequence data designating sample from fermenter as type. Several MOTUs which are potential

novel taxa are routinely discovered in fungal molecular ecology studies. The absence of true names for several uncultured sequences creates confusion and hence special sequence-based taxonomies are being developed[57]. The number of unclassified sequences is increasing in the on-line databases, which highlights the need for generating a code for such a nomenclatural system (as adopted by the prokaryote system). In an era of environmental sequence, it is one of the most important challenges for fungal taxonomy. Now high-throughput taxonomy is required for fast documentation of unknown fungal diversity[55]. However, in our view, instead of merging the MOTU with the present code of nomenclature, it should be kept separately as *Candidatus* species and should follow a separate set of rules. Merging both systems of nomenclature will de-mean the present system as 'basic' meaning of a species in both concepts is different. In one the type is a live culture and in the other type would be a sequence or an alignment. Maintaining quality of sequence is also an important aspect while designating the species in environmental sequence data. Hence, either *Candidatus* species, or eMOTU or ENAS (Environmental Nucleic Acid Sequence) should be considered as a separate code of nomenclature. Once any of its members is cultured later, it can be again described according to the present species concept by designating a type culture. This has occurred with *Archaeorhizomyces finlayi*, where a major clade of class Archaeorhizomycetes was known from environmental studies and a valid species was described when an isolate of *A. finlayi* was reported from conifer roots[59]. Similar example is provided by Rozellomycota, which contains the chytrid genus *Rozella*[60].

Specific-sequence regions and their length in metagenomic studies: Sequences such as 16S rRNA gene in Bacteria and ITS region in fungi have been routinely used in metagenomics studies as taxonomic markers. Use of different short-length (200–700 bp) regions from the 16S rRNA gene sequence in metagenomics studies and their extrapolation up to species level using bioinformatics tools are becoming more popular for diversity studies in environmental/ecological samples. Various regions from V1 to V9 in the 16S rRNA gene are used for amplification in bacterial diversity studies. Recommendations are made by Kim *et al.*[61] for clustering of species level OTU's at 0.03 distances from V1 to V3 regions of 16S rRNA gene for Archaea. However, in the case of Bacteria the V1–V3 and V1–V4 regions should be targeted, with species-level OTUs being clustered at 0.04 distance in both cases. This is only due to technological limitations in obtaining full-length sequences in community samples. Reliability and confidence in taxonomic status are always higher in full-length sequences. Hence for taxonomic purpose full-length sequence (~1400 bp) submission is a mandatory criterion set by the International Committee on Systematics of Prokaryotes.

For fungi, the ITS sequence is the official barcode region. Previously, to select a region for the second largest group of fungi which could identify environmental samples up to species level, other regions were also considered. These were SSU, LSU, *CO1* (cytochrome *c* oxidase subunit 1), etc. The SSU gene does not exhibit good differentiation power at the species level. Although *CO1* is used for species delineation of cultivable fungi such as *Penicillium* genus, Neocallimastigomycota and Oomycota clades, in environmental samples it is difficult to amplify due to inconsistency and cloning requirement[62]. For yeast, the D1 and D2 regions of LSU have been in use. Hence, for environmental samples usually a combination of ITS and LSU is used for species-level identification[63,64]. Phylogenetic tree construction has also been used, which is proving more effective[65,66]. Several protein-coding regions are being regularly used for species delineation in various fungal groups such as β-tubulin in *Penicillium* and EF1-α for *Fusarium*, but there is no report of any standardization for metagenomic studies using them. Schoch *et al.*[24] had demonstrated that ITS obtained more PCR success followed by LSU, SSU and RBP1. Almost 90–100% success was obtained for ITS of two phyla, i.e. Ascomycota (for Saccharomycotina, success was >90% for ITS, LSU, SSU) and Basidiomycota. Within ITS, it has been shown that ITS1 is on average somewhat more variable than ITS2 in most fungal lineages[67]. One should be clear in the concept of sequence length for taxonomic study and when working with environmental samples. The former is related to taxonomy and systematics (i.e. authentic identification of an organism) and the latter is related to annotation of sequences (for ease of interpretation of data), helping in understanding the functional and compositional characteristics of an ecosystem. The sequence length of ITS for taxonomy is the complete ITS region, 500–600 bp (ITS1-5.8S-ITS2), depending on the genera. For LSU, it may be D1 and D2 (300–400 bp) or the complete length (~1400 bp) and for environmental sequence it may be 100–300 bp.

Consensus in species concept

The methods of identification have changed from visible characters of differentiation to multi-gene phylogeny and biochemical analysis of proteins, metabolites, etc. (Figure 2). Apart from macroorganisms, we have been able to name and classify microorganisms. Staley[45] had discussed that prokaryotes and eukaryotes speciate in response to ecological and geographic factors through phylogenomic approaches. Therefore, a universal concept to define species can be developed. According to him, in order to develop a universal concept, bacteriologists will need to work closely with other microbiologists and biologists to reach an agreement on which species concept should be adopted. Hence, all species would be classified

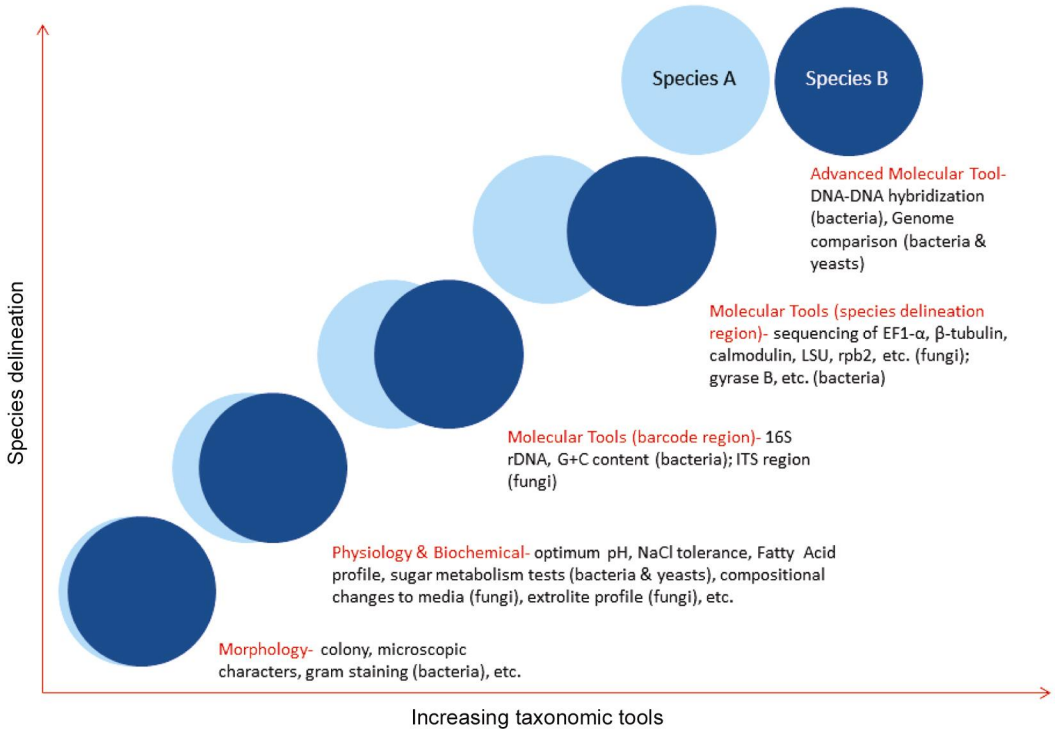

Figure 2. Different criteria used in species delineation of bacteria, archaea and fungi.

by the same criteria, which could help unite biology by completing the taxonomy of the entire Tree of Life from domain to species[45]. However, in our view, single-species concept cannot cover all organisms and is practically not possible. All biologists (including microbiologist) agree that morphological species concept (MSC) and biological species concept (BSC) should be abandoned and phylogenomic species concept (PSC) – smallest diagnosable monophyletic unit with a parenteral pattern of ancestry and descent, should be adopted[47].

There is no general agreement among microbiologists about what defines a species, especially in prokaryotes. Moreover, searching for a common concept is difficult due to the diversity and complexity of organisms and their genetic composition. Also, according to Paul[68], there is a need to understand the difference between species concept (species category) and species criteria (judging entity to species category). Hey[69] has pointed out that methods or protocols to identify a species should not be misunderstood or mixed with species concept, which is a usual scenario. Now phylogenomic concept may be taken up as a common concept with individual phyla exhibiting specific characteristics. However, it may soon change once whole genome-based sequencing methods are made mandatory for species identification. Efforts have already begun using average nucleotide identity (ANI)[70], which takes into consideration the whole genome sequence. Rokas *et al.*[71] have made a comparative study and species demarcation with genomes of different species of *Aspergillus*. Fitzpatrick *et al.*[72] have proposed a fungal phylogeny based on 42 complete genomes derived from super tree and combined gene analysis. Recently, Mende *et al.*[73] used whole-genome sequencing for species delineation and developed specI software (species identification tool; http://www.bork.embl.de/software/specI/). Other modern techniques such as matrix-assisted laser desorption/ionization (MALDI), time of flight mass spectrometry (TOF-MS), Raman spectroscopy are also used in the characterization of microbes (both bacteria and fungi), especially those that are medically important. Even if the whole genome is considered to be the criterion for species concept, substantial data need to be generated. Composition of whole genome in terms of functional and non-functional genes should also be considered for genome comparison.

In our view, nature is not bound by concepts. Concepts cannot cover the complete variety of microbes in nature. Kõljalg *et al.*[74] had expressed similar views that species recognition is not a simple matter and one size does not fit all. Moreover, concepts are ever changing with the discovery of new facts (which are already present since long), change in older techniques or discovery of high-resolution techniques and/or evolutionary changes. However, every new technique has a fixed-lifespan[75]. Direct comparison of species diversity in all three domains, i.e. Bacteria, Archaea and Eukarya is not possible since there are differences in how we define 'species' based on the criterion used. It will be always difficult to fulfil the need

of both geneticists and systematics in a single species concept. Moreover, microbes show speciation more rapidly than other complex eukaryotic organisms. Either there should be a separate species concept for different domains of organisms, or characters of microbes must also be considered while preparing a new modern species concept. It is critical to define the criteria as it can change the hierarchy of several taxa.

Concluding remarks

Observing a microbe during its whole life is also an important factor to record the changes in the morphological features. We use the term 'species' on several occasions, but cannot clearly define a species. At the end we ask a question, how can a species be resolved without consensus on what a species concept or what a species is? The word 'species' holds a different meaning for the evolutionary biologists compared to the morpho-taxonomists. It is a basic question for a school or an undergraduate teacher who has to teach about species to science students. It will help in nomenclature and classification of organisms, understanding of evolutionary trends, interaction between organisms in an ecological niche and properties associated with a particular organism. Moreover, it will be of great help to the microbial ecologists involved in community analysis, studying the composition, structure and function of microbial communities, whether it is in agriculture, grassland, water or forest ecosystem[76]. It is also necessary for studying community change in the ecosystem due to environmental/climate changes. Pathogenicity of a group of organisms (species) can be distinguished from a non-pathogenic species or strain, which is a boon in the field of diagnostics. In recent times, molecular sequencing has revolutionized microbial taxonomy. The data indicate enormous increase in the number of taxa published after sequencing of these regions began and the sequence database improved[70]. As observed in recent years, the next-generation sequencing (NGS) and its parallel technologies have drastically changed the metagenomics and in turn microbial taxonomy. One of the reasons for the same is reduction in the running cost of these technologies over the years (NGS, 454 sequencing, Illumina, etc.). There are at least three papers covering microbial taxonomy using whole-genome data in bacteria, fungi and algae[70,77,78]. Hence we believe that it is technology that will govern the criteria for identifying a species (directly or indirectly) and in turn the species concept. Moreover, in contrast to plants and animals, most of the microbial diversity is unsearched. Hence, species concept is bound to change with discovery of more microbial forms with unique characters (both at morphological and genetic levels). However, the final word lies with different commissions on taxonomy, be it the International Committee on Systematics of Prokaryotes or International Commission on the Taxonomy of Fungi. Such committees have helped the field of systematics (in particular) and ecology (in general) of various groups of organisms, keeping pace with new technologies and complexities in various taxonomical groups.

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