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Importance of Molecular and Phylogenetic Analyses for Identification of Basidiomycetes

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Abstract

Fungi are considered as diverse group of eukaryotic organisms and have very important role in ecosystem. Although their expected number is more than 2.2–3.8 million, only 120,000 taxa have been identified so far. Basidiomycetes are very large group of fungi including mushrooms, toad stools, puff balls, earth stars, polypores, and rust and smut fungi. Previously, these fungi were identified only by morphological characters that have been considered as variable due to environmental factors. Literature shows that many fungi are misidentified due to phenotypic changes. Molecular methods including phylogenetics prove to be successful aids along with traditional methods for correct identification of these fungi and these have revolutionized fungal reclassification. Many fungal taxa have been shifted to other groups of fungi after their phylogenetic analysis. So, many DNA markers can be used to solve such problems.

Keywords: Agaricales, morphology, mushrooms, primers, systematic

1. Introduction

1.1. Basidiomycetes

In biologist opinion, relationship of phylogenetics can be the dominant support of research in different areas of biology. The most expressing visions into biology are through species comparisons and phylogenetic analysis of gene sequence background. Its importance can be seen in diverse subfields including physiology, ecology, and molecular biology [1, 2].

The largest groups of fungi (Basidiomycetes) including many mushrooms, some are edible, have become more significant in recent times for their nutritional and medicinal properties. It is the second largest group of fungi that produce sexual basidiospores in modified cell called the basidium. This class has the resemblance with animal, plants, red and green algae, several groups of slime molds, water molds (oomycetes), brown algae, Ascomycetes (including lichens), and Phycomycetes (Glomeromycetes, Zygomycetes, and Chytridiomycetes) due to the presence of some important similar characters [3].

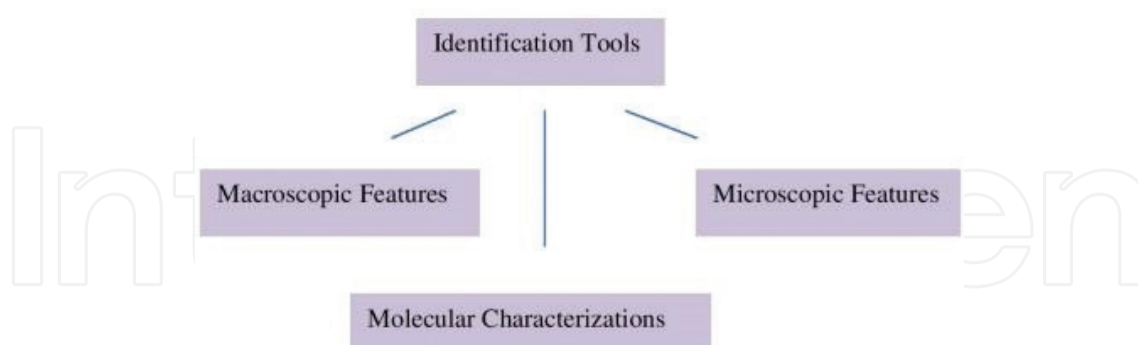
There are more than 30,000 species in Basidiomycota, and this number is increasing day by day [4]. More specifically, this division of characterization can be portrayed under the number of request of gilled and nongilled fungi [5]. Mueller and his companions [6] exhibited the aggregate expected number of gilled fungi around 80,000; out of which just 13,000 are known yet and these are extremely basic segment of forests, either on rotting wood and other dead plant material as saprotrophs or symbionts with the living cells of plant roots, forming mycorrhizal associations with trees, others are parasites on living plants [7].

1.2. Classification of Basidiomycetes

Basidiomycetes are categorized into rusts, smuts, Heterobasidiomycetes, Homobasidiomycetes, Gasteromycetes, Hymenomycetes, Dacrymycetales, Agaricales, and Aphyllophorales [8].

2. Methods

2.1. Cataloging techniques for Basidiomycete identification



Basically, scientists use three different markers for Basidiomycete identification including macroscopic, microscopic, and molecular analyses.

2.1.1. Macroscopic features for Basidiomycete identification

To be arranged appropriately, valid recognizable proof is required. There are numerous conventional techniques for distinguishing proof of these fungi, yet not every one of them are solid and reliable [9, 10]. Prior, the gilled fungi were recognized and named based on certain macroscopic features, that is, longevity, texture, color of internal tissues, form, spore and basidia bearing surface, dimensions, host and nature of deterioration accompanying with

a sporocarp on wood. Generally, Basidiomycetes (mostly mushrooms) are identified morphologically by their spore print color, ring and volva on stipe (presence/absence), substrate type, surface texture, and gill/hymenium attachment to the stipe. As we can observe that all these characters are variable to some extent with environmental conditions and cannot be used as prime features for identification purpose [11] (**Figure 1**).

2.1.2. Microscopic features for Basidiomycete identification

Traditionally, microscopic features are also used for the identification of these fungi [11]. Microscopic characters taken into consideration by many scientists include (a) hyphal composition of basidioma tissues which are of three types *viz.*, generative, skeletal, and binding hyphae. These hyphae form three different types of basidioma monomitic, dimittic, or trimitic; (b) nature of hymenium, basidia, cystidia, basidiospores, their shapes, dimensions, and color reaction in different reagents, and (c) clamp connections (presence/absence) [12] (**Figure 2**).

2.1.3. Misleading identification factors

The taxonomy of Basidiomycetes has been controversial because of the limited number of distinguish morphological characters, and there is uncertainty for sorting out of different sections and species. Environmental factors and substrate have great influence on phenotypic variation may cause troublesome in morphological identification of edible mushroom. One of the major issues for mushroom reproducers is the absence of an orderly consensus contrivance to segregate diverse species, which are occasionally morphologically indistinguishable [13].

Hence, they have to build up a proper strategy for distinguishing taxa [14]. The implements of molecular approaches are essential to confirm species delimitation. Traditional morphological strategies are less credible than cutting edge techniques that give more dependable approaches to distinguishing proof.



Figure 1. Some Basidiomycetes showing different morphological characters. The photos in the figure are the original collection by the authors of this chapter from Pakistan.

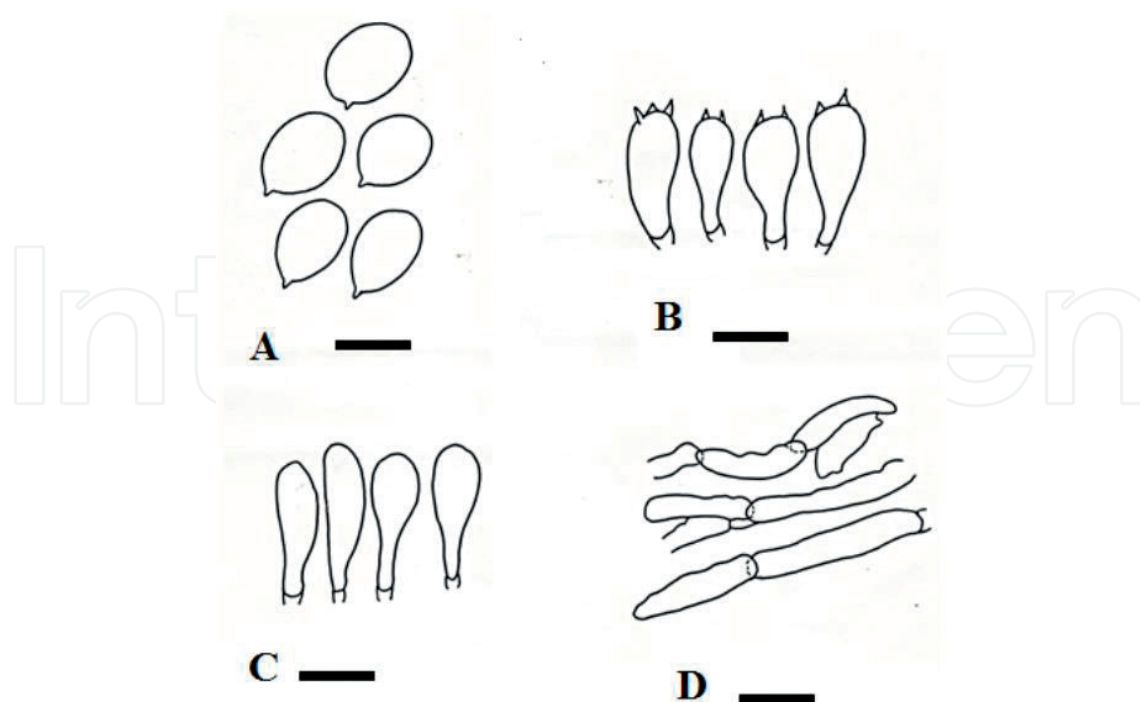


Figure 2. Different microscopic features of Basidiomycetes. (A) Basidiospores, (B) Basidia, (C) Cystidia, and (D) Pileipellis. These are the line drawings (anatomical structures) of *Agaricus* spp. prepared by the authors.

3. Advanced molecular methods for Basidiomycete identification

3.1. Molecular techniques

The recent improvement in DNA technology has been regarded as a prerequisite procedure provided a powerful addition to traditional taxonomic methods. Due to the limitations of conventional methods, molecular techniques are used to investigate the problems related to identification and classification of species. For fungal diagnosis, a high variety of molecular methods are progressively becoming important tools in all aspects for identification. There are several advanced level techniques that can be used for the identification of these fungi [15]. However, the use of DNA marker is base for all methods which provide connection between unknown fungi and fully described, morphologically characterized herbarium specimen. Fungal identification is somewhat dependent upon reference species that have been identified by mycological taxonomist for specific class of fungi that was taken into consideration with appropriate skills. Additional sources of information can be obtained from public DNA sequence databases for tentative identifications but should not totally relied upon these database sequences, as authenticating the distinctiveness of source material is rarely possible. Important molecular techniques include Southern blotting, PCR restriction fragment length polymorphism (PCR-RFLP), RAPD, PCR, DNA sequencing, microarrays, etc. DNA extraction and purification is the first step for any of these methods, for which many protocols and prepared kits are existing [16].

In fungal categorization, DNA strategies are fast and authentic to build up the individualities of wild collections. After the approach of cycle sequencing technique [16], direct sequencing of PCR products turned into a normal issue at least in organelle DNA loci or repetitive nuclear

DNA such as ribosomal DNAs [17]. This innovation is thought to be a standout among the most great techniques for phylogenetic investigations [18, 19]. Internal transcribed spacer (ITS) region of rDNA is usually utilized region for molecular recognizable proof of Basidiomycetes growing in differing natural surroundings. The corelationship among phenotypes and genotypes has been archived as phylogeny [20].

3.2. Fungal barcoding

A barcode is a categorization of a definite country of the genome which encompasses approximately genetic discrepancy among species, so countenancing one species to be renowned from an additional. The foremost DNA section which encounters this criterion for fungi is the “nuclear ribosomal internal transcribed spacer” or (ITS) expanse. Fungal DNA covers manifold copies of the ITS region which safeguards a virtuous resource of appropriate substantial for abstraction and examination. The barcode regions jumble-sale for fungal taxonomy characteristically ranges from 400 to 1000 base pairs in distance. Comprehensive studies which engender phylogenetic trees customarily expenditure arrangement evidence from supplementary than one barcode region. A barcode for an unidentified/unfamiliar species can be paralleled with barcodes apprehended in intercontinental records including GenBank and UNITE. Conversely, inaccuracies such as imprecisions in credentials of the original material or certification errors at a later date cast doubt on the validity of some records. A study by [21] nominated that more than 27% of all fungal ITS sequences were insufficiently identified in the International Nucleotide Sequence Database and in many cases had “compromised taxonomic annotations” [22].

3.2.1. Choice of primer

Choice of primer is a very crucial step. Nevertheless, one should start amplifying the ITS region of Basidiomycetes because of two reasons: first of all, universal primer for fungi (ITS1F) can work on it favorably, and secondly, this region has occupied maximum data of all type of fungi, incomparable to other barcoding regions which are now being the interest of scientist (Mycologist). Especially in the case of nom. prov. (seems new) species where data based on one genetic region seems insufficient and unreliable. Moreover, the most suitable primer will be chosen according to the category of a Basidiomycete to which it belongs to. Mostly, universal primer for fungi, that is, ITS1F is used as a forward primer that reads from 5' to 3' direction of one template strand, while ITS4 is being used as reverse primer that reads the second template DNA strand from 3' to 5' direction. There are many other fungal specified primers that have been used for different groups of fungi [9] (**Figures 3 and 4**).

3.2.2. Fungal barcoding primers

Following are some important primers that are under the use for molecular and phylogenetic study of Basidiomycetes.

- ITS Primers: ITS1, ITS2, ITS3, ITS1F, ITS4, ITS8-F, ITS6-R, ITS4BR, ITS4BR2, ITS3R2, ITS242, ITS5, ITS3R3, 5.8S, 5.8SR, UN-UP18S42, UN-LO28S22, BE1, and BE2.
- LSU Primers: LR0R, LR5, and LR16.
- SSU Primers: SR1R, NS1, NSR, PNS1, and NS41.

- RPB1Primers: RPB1-Af, RPB1-Ac, and RPB1-Cr.
- RPB2 Primers: fRbB2-5F, RPB2-7R, and Brpb2-7.1R.
- MCM7 Primers: Mcm7-709for, Mcm7-1348rev, and Mcm7-1447rev.

3.2.3. Phylogenetics

Phylogenetics is the learning of evolutionary associations among biological bodies often genes, individual or species and assists to classify the organism, finding pathogenies, forensic sciences or in bioinformatics. Sometimes, it provides base line to investigate the fundamental relationships among different taxa belonging to whether same or different class, while most of the time, it also helps in approaching application of a particular morphon [23].

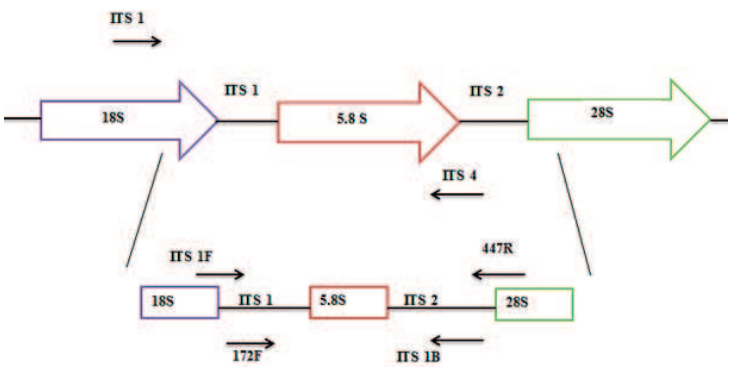


Figure 3. Internal specified region of a part of genome. © Mishra RK, Verma DK, Pandey BK, Pathak N and Zeeshan M (2014) Direct Colony Nested-PCR for the Detection of *Fusarium oxysporum* f. sp. *Psidii* Causing Wilt Disease in *Psidium guajava* L. J Horticulture 1:105. doi:10.4172/2376-0354.1000105.

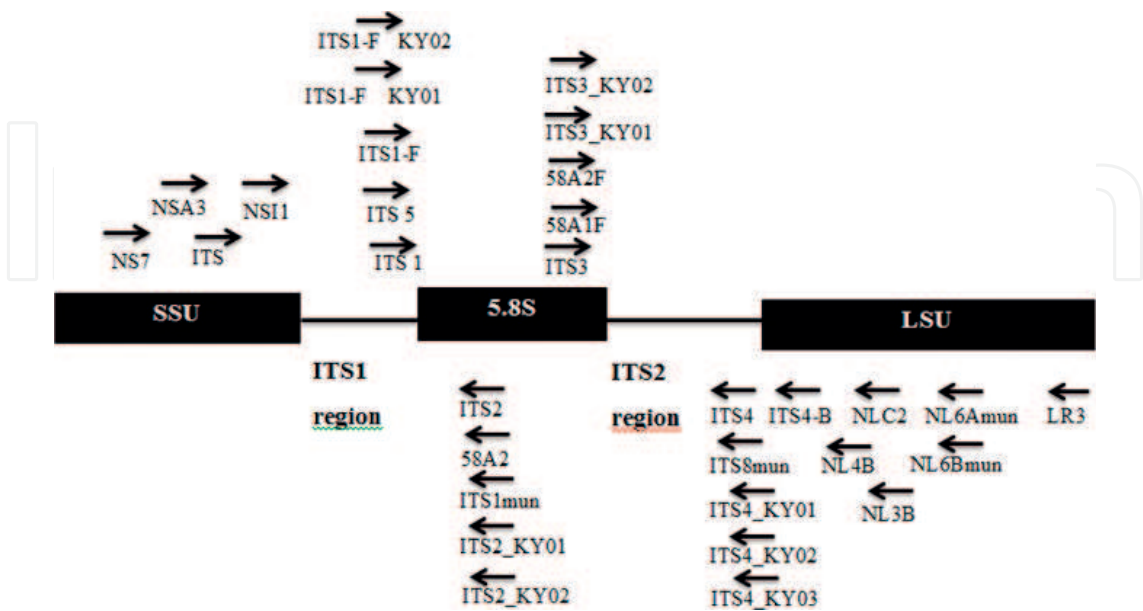


Figure 4. Three regions and their directions to amplify. © Toju H, Tanabe AS, Yamamoto S, Sato H (2012) High-Coverage ITS Primers for the DNA-Based Identification of Ascomycetes and Basidiomycetes in Environmental Samples. PLoS ONE 7(7): e40863. <https://doi.org/10.1371/journal.pone.0040863> credited.

4. Results and discussion

4.1. Example for basidiocarp identification (problem solving)

Entoloma rhodopolium is a poisonous species causes gastrointestinal diseases, and muscarine, muscardine, and choline have also been insulated as noxious mediators. It is commonly known as wood pink gill often confused with morphologically similar species *E. sarcopum* (edible). To save someone's life, correct and authentic identification is very much necessary here. Hence, finally phylogenetic investigation of *E. rhodopolium* was accompanied by using RPB2 and ITS sequences, and the result was matched with that of previously described species from Europe making three clades. Based on the taxonomy, a simple proof for the identification technique, PCR-RFLP was followed to distinguish between edible *E. sarcopum* and poisonous species which was actual parallel in morphology. The learning can provide assistance to elucidate the classification of complex *E. rhodopolium*-related species, and to take avoiding action from food poisoning [17] (Figure 5).

Similarly, Nawaz et al. [24] carried out a research to identify *Melanoleuca* species from Pakistan. Only morphological parameters cannot help to identify of *Melanoleuca* species [25, 26], and so, their identification mainly depends on phylogenetic analyses [27]. *Melanoleuca dirensis* is distinct from the other taxa in the subgenus based on the morphoanatomical and phylogenetic characters. Although, the size of the stipe and lageniform cystidia are shared characters between *M. cinereifolia* and *M. dirensis*, *M. dirensis* differs from *M. cinereifolia* in having white lamellae and fusoid-ventricose cheilocystidia, while *M. cinereifolia* bears gray lamellae [25, 27]. *Melanoleuca dirensis*, a new species from Pakistan [24] belonging to above mentioned genus was identified by phylogenetic tree analyses.

4.2. Example for ectomycorrhizal morphotype identification

Ectomycorrhizal association of Basidiomycetes is an important part of any ecosystem for trees growth which leads toward increase in forestry. Previously, ectomycorrhizal morphotypes



Figure 5. *Entoloma rhodopolium* (copyright) of Michael Kuo (Kuo, M. (2014, January). *Entoloma rhodopolium*. Retrieved from the MushroomExpert.Com).

were identified by morphotyping methods [28]. No doubt, characters for morphotyping are important for the identification and taxonomic purpose, but some time these characters mislead in identification [29] due to similar characters in different morphotypes and different characters of same species morphotypes when their host tree is different. Molecular and phylogenetic analyses resolve this problem. Now mycologists can easily identify mycobiont as well as phycobiont by using such advanced methods. Corresponding author of this chapter has identified many mycobionts from Himalayan range of Pakistan by using molecular methods [30–34]. Following phylogenetic trees are two examples among these. **Figure 6** explains ectomycorrhizal morphotypes of *Suillus flavidus*. These morphotypes were isolated from rhizosphere of conifers from Pakistan and were tried to identify by morphotyping methods, but ultimate identification was possible only by molecular and phylogenetic analyses [32]. Similarly, **Figure 7** explains mycobiont of another mushroom *Suillus himalayensis*,

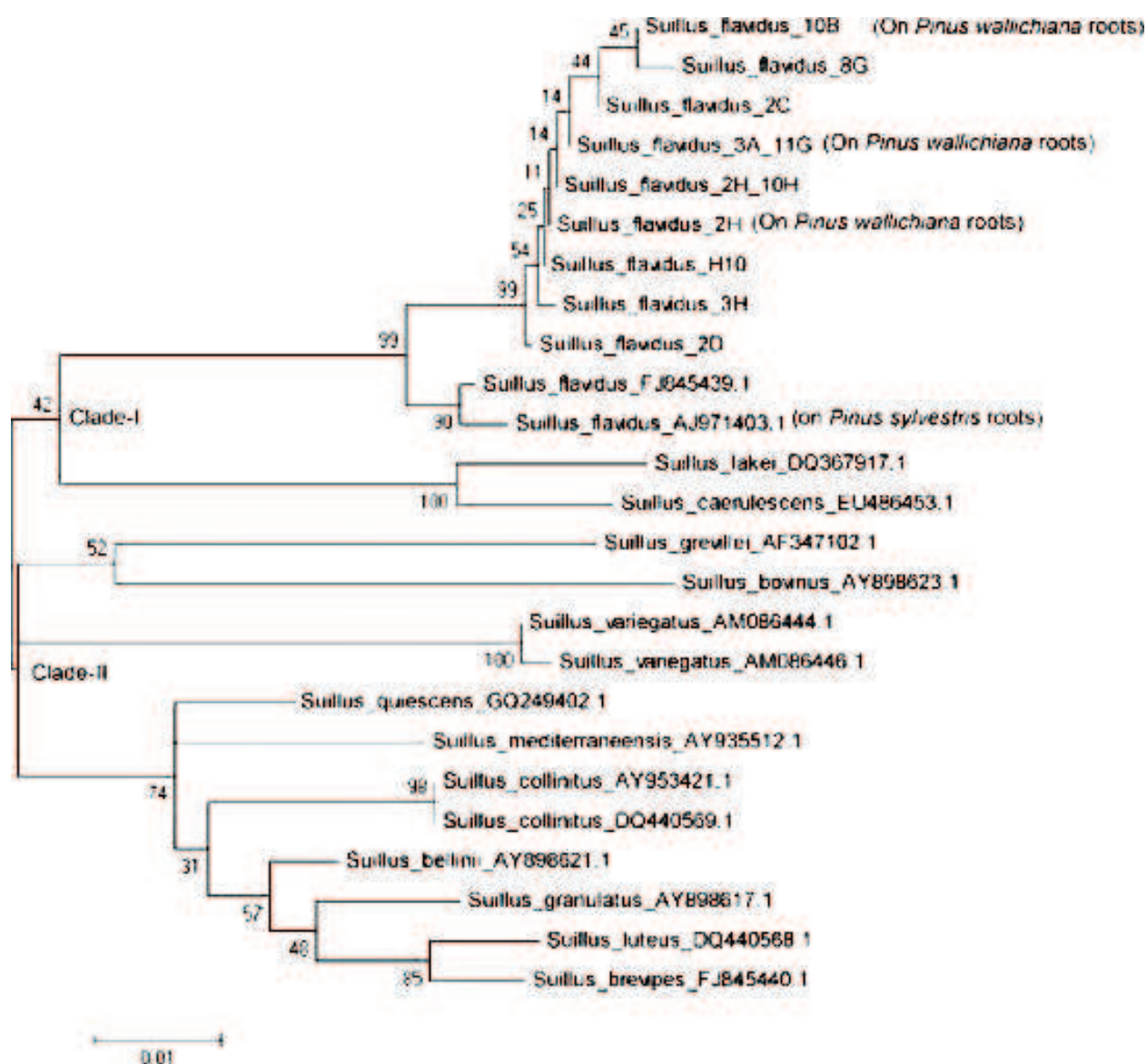


Figure 6. Phylogenetic analyses of ectomycorrhizal morphotypes of *Suillus flavidus* [32].

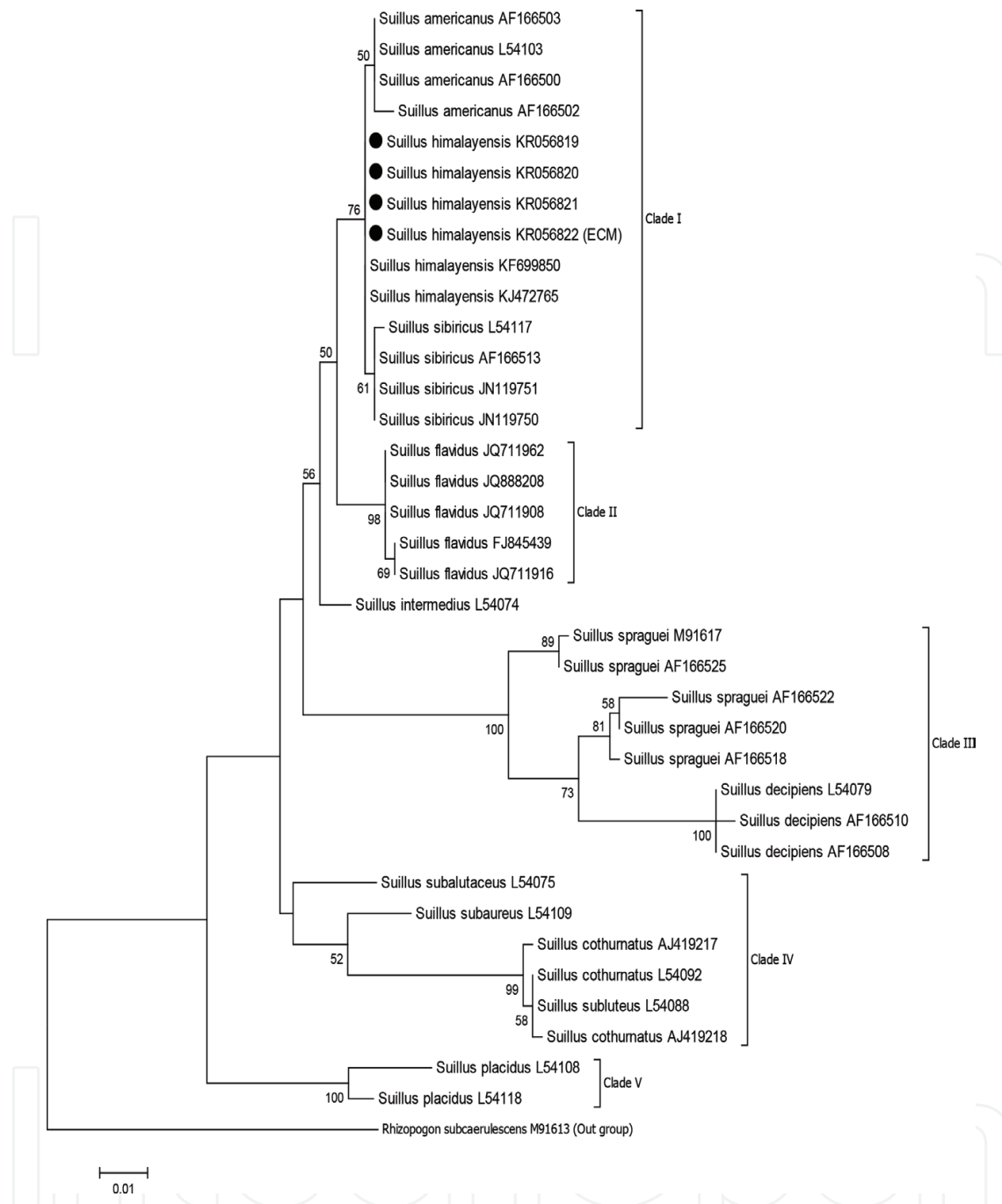


Figure 7. Phylogenetic place of *Suillus himalayensis* [34].

a new species reported from Pakistan by corresponding author. Its ectomycorrhizal relationship was confirmed when morphotypes were analyzed phylogenetically [34].

5. Problems that need to be addressed

The absence of sequences at a local level would be a chief hindrance for the recognition of some Basidiomycetes. Robles et al. [35] worked to analyze the scope of facts

attained from ITS sequences as taxonomic implements to inspect local wood-rotting fungi. Phylogenetic analyses were made under static and vibrant homologies, but identification of some of these fungi was not attained due to the intricacy of the genera and the deficit of sequences [35].

Another fungus *LeucoCalocybe mongolica* has application in food industry and atmosphere investigation, is a noteworthy unusual wild edible mushroom in Northeast Asia. Its genomic sequence is vital to be studied at genus and species level in taxonomic classification. Beyond that, there is limitation in further study by virtue of the way that transcriptomic and genomic information of *L. mongolica* lacked in the biological information database. For such investigation, the transcriptome information is accomplished by virtue of Illumina paired-end sequencing innovation [36].

For taxonomic identification of Basidiomycetes, the sequence of the ITS region is a superior molecular DNA barcode [37]. As most of the studies so far done to identify the fungal species has used primers (forward and reverse) against this most highly varied region to amplify. Most of the times partial rDNA sequences, including the Internal Transcribed Spacer I-5.8SrDNA-Internal Transcribed Spacer II, are used, and further phylogenetic assessments are made to see relationships between edible species of the Basidiomycetes. Polymorphism occurred due to insertion-deletion and point mutations throughout the ITS regions and can be clearly distinguished within genera as well as families [38].

5.1. Why practice molecular documents?

Today, virtually all evolutionary interactions are contingent from molecular sequence data. This is because:

- DNA is the congenital material;
- We can here and now effortlessly, hastily, economically, and dependably sequence genetic substantial;
- Sequences are extremely specific and are often facts rich.

Morphological lineages are also made where genetic lineages are not possible (e.g., in few fossil records), but they are not reliable as we discern that every now and then the similar morphological mannerism can ascend from manifold independent evolutionary lineages.

5.2. Stages

1. Start with a question; which is the identification of a basidiomycete at species or genus level.
2. Identify a model and parameters that could answer the question.

3. Collect sequence data that would help to answer the question.
4. Identify the orthologous sequences.
5. Align sequences.
6. Estimate tree and other parameters given the data and model.
7. Estimate the error associated with the tree and/or parameter estimates.
8. Does it answer your question?

5.3. Phylogenetic resources at EMBL-EBI

EMBL-EBI offers a range of tools and resources that are relevant to the field of phylogenetics:

- Ensembl fungi are a vast resource for fungal genome data.
- Ensembl genomes extends Ensembl across the tree of life, making genome data publically available for bacteria, plants, fungi, protists, and metazoa. This includes pre-computed alignments and orthologues.
- Ensembl compara offers pre-computed phylogenies for visualization and download.
- ClustalW2 Phylogeny is a basic tool for estimating evolutionary trees from multiple sequence alignments. It uses the Neighbor Joining method with the option of a very simple model of sequence evolution [39].
- EMBOSS Seqret is a file format conversion tool that can be useful at multiple stages of a phylogenetics workflow.

After performing the first initial BLAST, a phylogenetic tree is produced using different software, for example, different versions of MEGA and SYPRUS (**Figure 8**).

5.4. Explanation of the figure obtained by using MEGA 6 software for molecular characterization and phylogenetic analysis of *Coprinopsis* species

After morphoanatomical characterization of *Coprinopsis* species gathered from plain territories of Pakistan, it was considered for molecular affirmation. Sequence brought about 1070 bp of their ITS region. The sequence was gone intensive BLAST search. Introductory BLAST investigation indicated 99% match with *C. cinerea* (AB097562). In addition, comparative groupings were likewise incorporated into this phylogeny. The entire informational collection involves 32 nucleotide sequences comprising 701 positions. The phylogenetic tree for *Coprinopsis* with sequences from Genbank was separated in four clades. *Coprinopsis cinerea* (BIF S21) falls in Clade I in *Cinerea* section making bunched with other *C. cinerea* species of different countries.

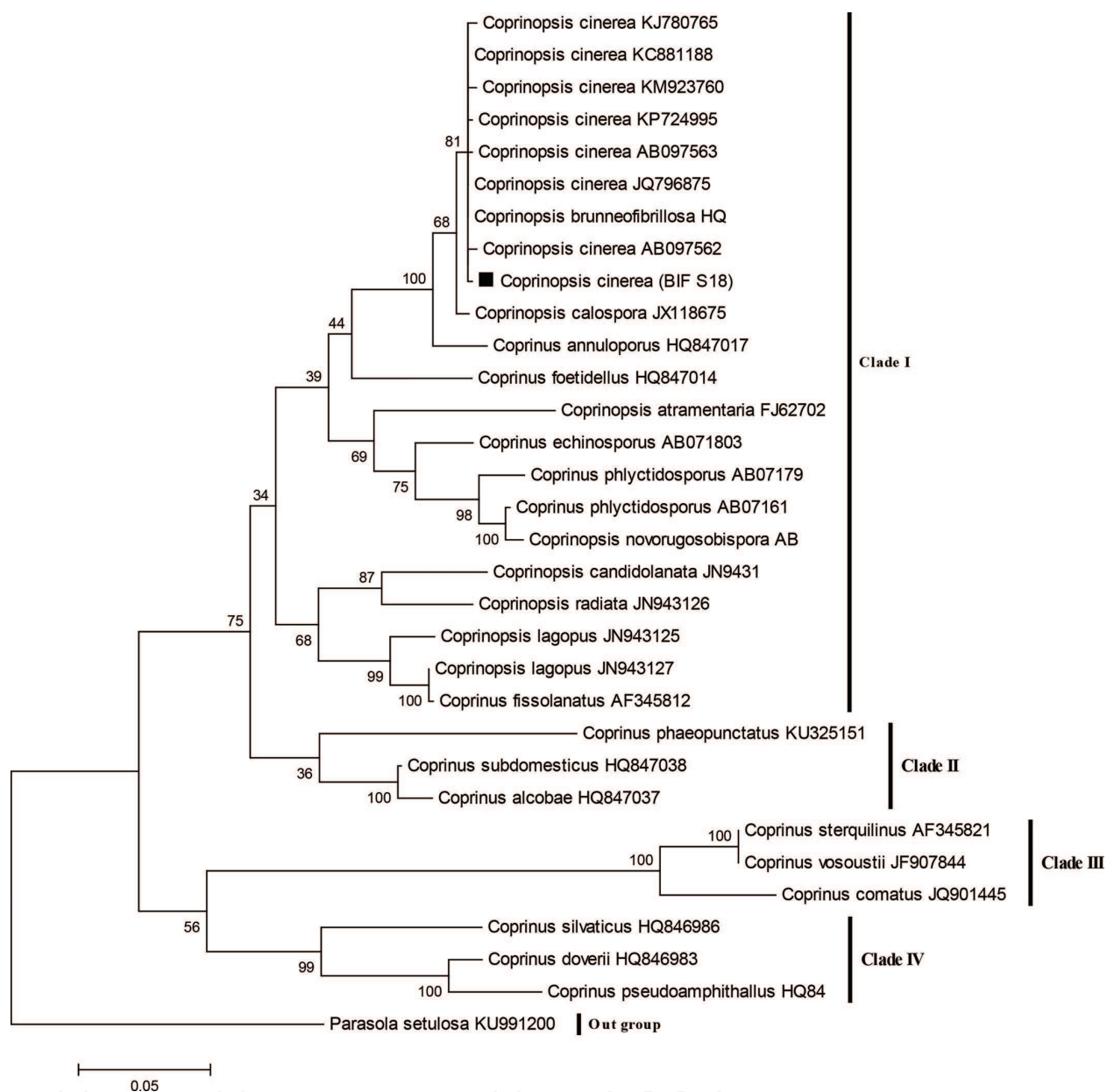


Figure 8. Phylogenetic analysis of *Coprinus* species collected from Pakistan based on nrITSr-DNA regions. This is the original phylogenetic tree made by one of the author of this chapter.

6. Conclusion

Basidiomycete is an important group of fungi that includes fungi forming ectomycorrhizae with trees, edible and medicinally important mushrooms, saprotrophs of wood and leaf litter, etc. and pathogens causing tree decline, wilting, and rots. Most of these have been identified and divided by morphological basis till eighteenth century by Friesian system, that is, all gilled fungi were included in Agaricales, all nongilled fungi in Aphyllorphorales, and all macrofungi with internal spore production in Gasteromycetes. Molecular methods using DNA extraction, amplification of a specific target region, and sequencing have confirmed to be more steadfast methods of identification. Molecular and phylogenetic characters have

resolved many controversies. Although classical methods are useful for enlisting species of a particular area, these methods for fungal identification alone cannot work better due to phenotypic variations. Combining classical approach with molecular and phylogenetic techniques is an appropriate way for identification, taxonomic, and purposes.

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