



Unraveling Phylogenetic Relationships via *A. carbonarius* and *A. tubingensis* Sequence Analyses [†]

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Abstract: The aspergilli comprise a diverse group of filamentous fungi spanning over 200 million years of evolution. In this work, we report a clarification of the phylogenetic relationship between *A. carbonarius* and *A. tubingensis* via the screening of ITS-5.8rDNA, (niiA-niaD), β -tubulin, and eEF-1 genes. The phylogenetic analysis of aspergilli ITS-5.8rDNA sequences divided *A. carbonarius* and *A. tubingensis* into two coherent clusters and showed a close intergeneric relationship, which is in keeping with the existing morphological and taxonomic classification. Herein, *A. carbonarius* exhibits a complete similarity (100%) for the three target genes: intergenic region of (niiA-niaD), β -tubulin, and eEF-1. The tree resulting from a bootstrap analysis indicates branch points when the significance is 98% minimum for each gene (β -tubulin and eEF-1). The phylogenetic analysis of the aspergilli genome structure provided a quantitative evaluation of the forces driving long-term eukaryotic genome evolution. It also led to an experimentally validated model of mating-type locus evolution, suggesting the potential for sexual reproduction in *A. carbonarius* and *A. tubingensis*. This is the first work describing the evolutionary relationship between both species. Nevertheless, this discovery might be viewed as typical during the process of new taxonomic evolution.

Keywords: *Aspergillus carbonarius*; *Aspergillus tubingensis*; β -tubulin; eEF-1; ITS-5.8rDNA; niiA-niaD; phylogenetic analysis



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1. Introduction

The *Aspergillus* genus is widespread in nature [1–4], and many species of this genus have significant negative effects on agriculture, particularly the production of mycotoxins such as ochratoxin A [1,5,6]. The filamentous fungi *A. carbonarius* and *A. tubingensis* have been extensively employed in genetic research, and toxigenic potential studies can be carried out on them [6]. The concern about these filamentous fungi in the vineyard has traditionally been linked to spoilage of grapes due to fungal growth. Nevertheless, these two saprobic fungi can produce mycotoxins in grapes [7–9]. The phenotypic concept is the classic approach based on the morphological characteristics suggesting that both species are morphologically far [10–12].

Partly due to its economic importance, the genus *Aspergillus* has one of the better classifications among filamentous fungi [11,13,14]. Raper and Fennell, in 1965, described 18 species groups within this genus based mainly on cultural and morphological features [8]. These were treated as sections belonging to six subgenera by Gams et al., 1985 [15]. Phylogenetic studies of ribosomal RNA gene sequences led to the acceptance of 3 subgenera with a total of 15 sections [16]. In the present work, considerable attention was devoted

to developing molecular tools to give a general overview of the taxonomic relationships between *A. carbonarius* and *A. tubingensis* based mainly on our recent studies.

The techniques applied included PCR-based methods and phylogenetic analysis of ITS-5.8rDNA (*niiA-niaD*), β -tubulin, and eEF-1 gene sequences in comparison with morphological features.

2. Methods

2.1. Organisms, Growth Conditions, and DNA Isolation

It is crucial for practical purposes that fungi are isolated and correctly identified. By appropriately adjusting the temperature and medium composition, it is possible to preserve the two morphological stages of fungal species in a lab setting; mycelia cells developed in vitro at a temperature of 25 °C in the dark. Strains from Tunisian grapes were employed in this investigation. *A. carbonarius* CBS 120167 and *A. tubingensis* strain CBS 11732 were used and collected from the American Type Culture Collection (ATCC) and employed as references. For subsequent uses, mycelia were collected by filtration via filter paper or Miracloth (Calbiochem, Darmstadt, Germany), frozen in liquid nitrogen, crushed, and kept at –80 °C. Using the Fungal DNA miniprep kit (QIAGEN-France), fungal DNA was extracted from frozen mycelial powder in accordance with the manufacturer's instructions. The DNA levels were measured using a NanoDrop® ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, NC, USA) at 260 nm and adjusted to approximately 25 ng/ μ L.

2.2. PCR Amplification, Cloning and Sequencing

The PCR amplification of ITS-5.8rDNA, the intergenic region of (*niiA-niaD*), β -tub, and eEF1 genes were run in a "GeneAmp PCR 2700" thermal-cycler (AB-Applied Biosystem, CA, USA). PCR reactions were performed using the Hot Gold Star Mix (Eurogentec, Belgium). Fungal genomic DNA was used as a template to target the ITS-5.8rDNA, the intergenic region of (*niiA-niaD*), β -tub, and *eF1* genes in a 50 μ L reaction mixture containing 25 μ L Hot Gold Star Mix, 1.2 μ L of each primer (PN1-PN34, Nit 462F-Nit 873R, β tub- β tubR), and eEF1-1526F-eEF1-1567R. The reactions were performed with the following program: initial denaturation at 95 °C for 1 min; 35 cycles, and each cycle consisted of three steps of 95 °C for 1 min; an annealing temperature (X) °C (Table 1) for ITS- 5.8rDNA, (*niiA-niaD*), eEF1, and β -tub; and a last step at 72 °C for 1 min followed by a final incubation at 72 °C for 10 min. The products amplified were separated via horizontal electrophoresis on 2% agarose gel using Tris–Boron–EDTA buffer (TBE) 0.1X for 1 h at 100 V. Fragment size was estimated using a suitable molecular marker (Smart DNA Ladder, Eurogentec, Belgium), and the PCR products were purified using PEG (Polyethylene Glycol) and purification spin-columns.

Table 1. Primers used in the study.

Primer Code	Primer Sequence (5' to 3')	Annealing Temperature (X) °C
PN1 PN34	AGTAAAAGTCGTAACAAGG TTGCCGCTTCACTCGCCGT	55
PU PR	CGTTGTAAACGACGGCCAGT GTACCAGTATCGACAAAGGAG	52
Nit-462F Nit-873R	GTTACGGAAGCAAAGAGGTA GTTATTAGGGGCTATGGCAC	54
β -tubF β -tubR	CTCGAGCGTAGTAACGCTCTAC AAACCCTGGAGGCAGTCGC	55
eEF1-526F eEF1-1567R	GTCGYGTYATYGGHCAYGT ACHGT RCC RATAACCACC RAT CTT	58

The clones amplified by PCR were subcloned into the *pGEMT* vector (Promega) and, after heat shock, cloned in chemically competent Top10 one shot® cells of *Escherichia coli* (Invitrogen, Karlsruhe, Germany). The primers PU-PR were used for cycle sequencing in a programmed GeneAmp PCR 2700 thermal-cycler (AB-Applied Biosystem, CA, USA).

(Table 1) as insert-specific primers in the vector “pGEMT” with the following temperature profile: 20 s at 96 °C, 20 s at 50 °C, 4 min at 60 °C, for 30 cycles. The CEQTM 8000 Genetic Analysis System was used to perform the sequencing process.

2.3. Sequence Alignment and Phylogenetic Analyses

Nucleotide sequences were determined using sequence data, and predictions of the protein structure were analyzed using the Clustal W method with the MegAlign program of DNASTAR software (Lasergene, WI, USA).

3. Results

3.1. Screening of ITS-5.8 S rDNA in *A. carbonarius* and *A. tubingensis*

Interspecific variability of *Aspergillus* species assigned to section *Nigri* was also examined using phenotypic features and sequences of the intergenic transcribed spacer regions (ITS region) and the 5.8 S rDNA gene (Figure 1). The phylogenetic analysis of sequence data indicated that *A. carbonarius* and *A. tubingensis* are presented in two distinct groups. This result was also supported by phenotypic data.

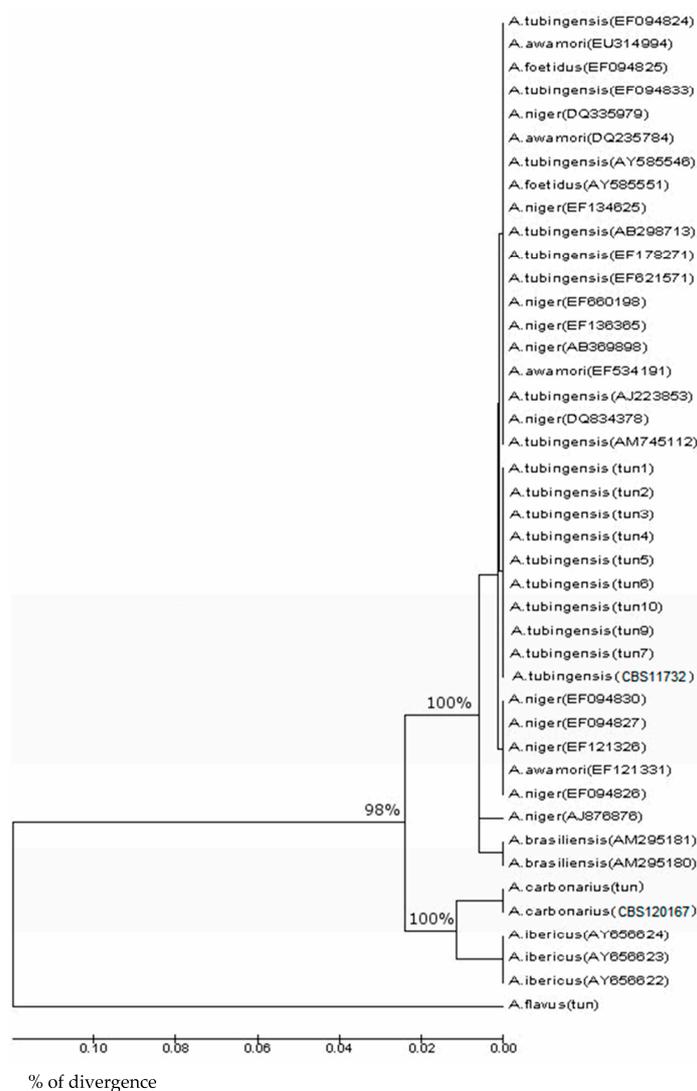


Figure 1. Neighbor-joining tree of ITS sequences of species assigned to *Aspergillus* section *Nigri*, including *A. niger*, *A. tubingensis*, *A. awamori*, *A. foetidus*, and *A. brasiliensis* clustered in the same branch. Positive control *A. flavus* was employed, and *A. carbonarius* and *A. ibericus* were grouped together.

The uniseriate species *A. carbonarius* and *A. ibericus* are clustered together and defined in a second clade, while *A. awamori*, *A. foetidus*, *A. tubingensis*, *A. niger*, and *A. brasiliensis* are on a separate branch with respect to other black *Aspergillus* species, according to the phylogenetic analysis of ITS sequence data. At least several species belong to this section. The last *Aspergillus* strain, *A. flavus*, was used as a control (Figure 1).

3.2. Screening and Phylogenetic Analysis of (*niiA-niaD*) Gene in *A. carbonarius* and *A. tubingensis*

The intergenic portion of the (*niiA-niaD*) gene was isolated within *A. carbonarius* and *A. tubingensis*. The nucleotide sequence suggests that *A. carbonarius* has two distinct DNA fragments; one of these (400 pb) was also amplified in *A. tubingensis* (Figure 2).

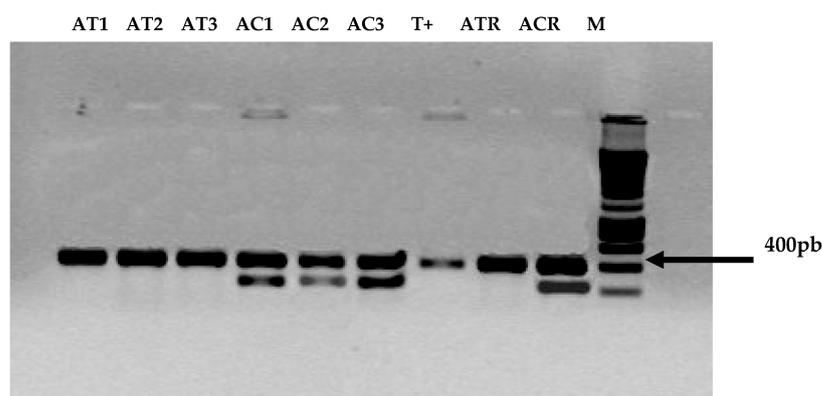


Figure 2. Results of the PCR used to target 400 pb of the intergenic portion of (*niiA-niaD*). From the left to the right (AT1-AT3): *A. tubingensis* isolates from grapes; AC1-AC3: *A. carbonarius* isolates; T+: positive control; ATR: *A. tubingensis* CBS11732 (reference strain); ACR: *A. carbonarius* CBS 120167 (reference strain) and M: Size DNA markers.

The alignment and the comparison of the two sequences suggest a full homology between both *Aspergillus* species. Following this result, a hypothesis was expressed about the existence of a phylogenetic relationship between both species. To confirm this hypothesis, two genes were targeted: β -tubulin and eEF-1.

3.3. Screening and Phylogenetic Analysis of β -Tubulin Sequences

The β -tubulin gene was used to probe the genomic DNA of *A. carbonarius* and *A. tubingensis*. The alignment of the β -tubulin genes isolated from *A. carbonarius*, *A. tubingensis*, *A. carbonarius* (CBS 120167), and *A. tubingensis* (CBS 11732) were used as a reference strain and gave evidence for a high degree of similarity (100%). In addition, the amino acid sequence deduced from the nucleotide sequence of β -tubulin was used to develop a phylogenetic tree which indicates that *A. carbonarius* and *A. tubingensis* were clustered together in the same branch with amino acid sequences of *A. tubingensis* (DQ902579) and *A. carbonarius* (AY585532).

Isolates of *A. carbonarius* (AY585532), *A. tubingensis* (DQ902579), *A. tubingensis* 1–2, *A. tubingensis* (CBS 11732), *A. carbonarius* (CBS120167), and *A. carbonarius* 1–2 are clustered in group A. *A. foetidus* (DQ768454) and *A. costaricensis* (AY820014) are clustered in group B. Group C is composed of two branches with *A. brasiliensis* (DQ900611), *A. niger* (AY80001), (AY585535), and *A. lacticoffeatus* (AM419748). The last group consists of *A. ibericus* (AM419748) and *A. sclerotioniger* (AY819996).

The tree results from a bootstrap analysis that indicates branch points when the significance is 98% minimum for *A. tubingensis* and *A. carbonarius* species (Figure 3). Our results suggest that the phylogenetic analysis of the β -tubulin DNA sequence strongly supported the hypothesis.

3.4. Screening and Phylogenetic Analysis of eEF-1 Sequences

The alignment of eEF-1 sequences showed a full similarity between *Aspergillus* species (*A. carbonarius*, *A. carbonarius* (CBS 120167), *A. tubingensis*, and *A. tubingensis* (CBS 11732)).

Two distinct groups within the eEF-1 sequences were obtained for the four isolates of *Ascomycota* (*Aspergillus carbonarius*, *tubingensis*, *oryzae*, and *Trichoderma gamsii*). Isolates within group A were separated from those in group B by approximately 15 base mutations.

The phylogenetic tree results from a bootstrap analysis that indicates branch points when the significance is 98% minimum within group A compared to 75% across isolates within group B (Figure 4).

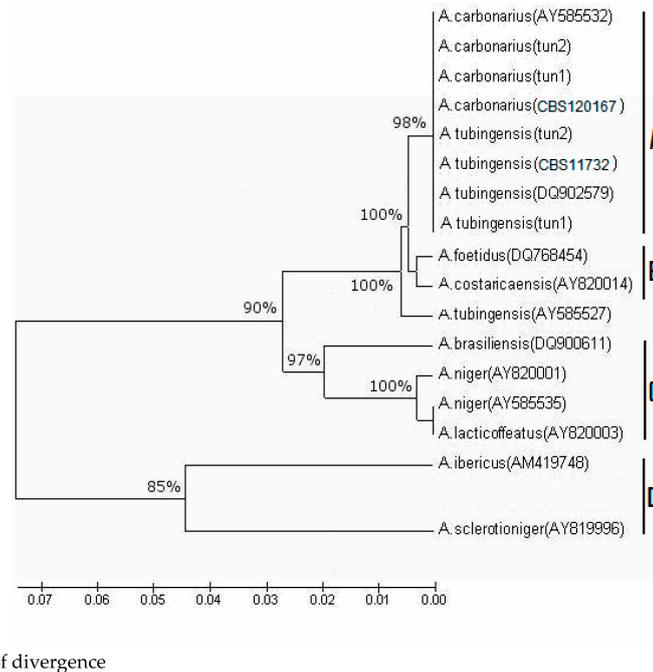


Figure 3. Phylogenetic tree based on amino acid sequences of β -tubulin within *Ascomycetes*: *A. carbonarius* (AY585532), *A. tubingensis* (DQ902579), *A. tubingensis* 1–2, *A. tubingensis* (CBS 11732), *A. carbonarius* (CBS 120167), and *A. carbonarius* 1–2 are clustered in group (A); *A. foetidus* (DQ768454) and *A. costaricensis* (AY820014) are clustered in group (B) and related to group (A). The third group, (C), is composed of two branches containing *A. brasiliensis* (DQ900611), *A. niger* (AY80001 and AY585535), and *A. lacticoffeatus* (AM419748), and the group (D) consists of *A. ibericus* (AM419748) and *A. sclerotiumniger* (AY819996). Bootstrap percentages were calculated and ranged from 85% to 100%.

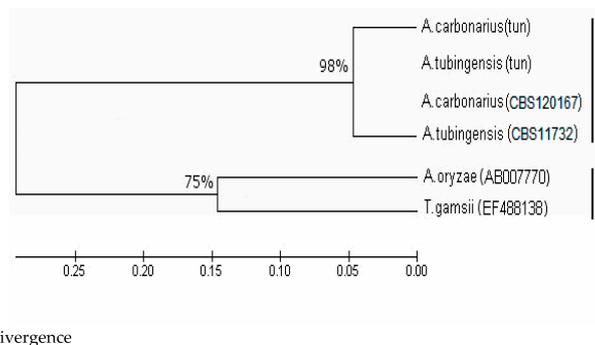


Figure 4. Phylogenetic tree using eEF-1 amino acid sequences of *Ascomycetes* (*A. carbonarius*, *A. tubingensis*, *A. oryzae* (AB007770), and *Trichoderma gamsii* (EF488138)). Bootstrap percentages were calculated and are indicated at nodes 98% for group (A) (*A. carbonarius* and *A. tubingensis* isolated from Tunisian grapes, *A. carbonarius* (CBS 120167), and *A. tubingensis* (CBS 11732)) and 75% for group (B) (*A. oryzae* and *T. gamsii*).

The amino acid sequences were evident from both the visual inspection of the aligned sequences and the dendrogram produced via an analysis of the sequence data. The identification of an evolutionary link between the two species was highly confirmed by DNA sequences from β -tubulin and eEF-1, supporting the hypothesis.

4. Discussion

Modern molecular tools used to identify phylogenetic relationships between fungi have opened new possibilities for expanding insight into and knowledge of this area. In recent years, the use of multiple gene analyses for phylogenetic studies has become more popular, and several studies based on protein-coding genes have been published, including fungal taxa [17–19]. De Jong et al. (2001) showed that the analysis of multiple loci can provide more resolution and therefore more reliable phylogenetic hypotheses in fungal phylogeny. The phylogenetic analysis of sequences of the ribosomal ITS-5.8rDNA gene cluster and the β -tubulin gene were found to be very useful for clarifying taxonomic relationships among toxigenic aspergilla [2].

In this report, ITS-5.8rDNA was used for *A. carbonarius* and *A. tubingensis*, and analysis led to two far distinguished species. In fact, previous work based on large subunit rDNA data has led to a widely accepted phylogeny of the aspergilli in which *A. tubingensis* and *A. niger* are more related to one another than *A. carbonarius* [4,20]. However, single-gene phylogenies can contradict organismal phylogenies. In principle, whole-genome data provide greater resolving power by allowing trees to be constructed based on concatenated sets of genes. Using this approach to study the relationship of both aspergilli (*A. tubingensis* and *A. carbonarius*), we find support for an alternative phylogeny. In the current study, we reveal that 400 bp of the target gene (*niiA-niaD*) are subjected to a conserved sequence that separates *A. tubingensis* from *A. carbonarius*. PCR assay for (*niiA-niaD*) was conducted to a hypothesis of an evolution relationship. In disagreement with our previous work, the intergenic region of (*niiA-niaD*) was used to distinguish between *A. niger* and *A. tubingensis*, and two primer sets were designed for each species [6,13]. Here, our results based on sequence alignment and the phylogenetic analysis of the eEF-1 and β -tubulin strongly supported the recognition of this process of evolution within *A. carbonarius* and *A. tubingensis*.

According to many studies, the genes for tubulins, especially for β -tubulin, are receiving increasing attention in the investigation of evolutionary relationships at all levels: (i) in kingdom-level phylogenetic analyses [17,21] and (ii) in studies of complex species groups within protists, animals, fungi, and plants [13,22–24].

In agreement with several studies, β -tubulin was usually used to detect evolutions in fungal species [18,21].

Our hypothetical dataset is also used to demonstrate how genealogical relationships and population parameters can be inferred using PCR methods.

The basic principles of these approaches are graphically presented by phylogenetic trees, along with useful references such as *A. tubingensis* CBS 1732, *A. carbonarius* CBS 120167 and comments on key assumptions implicit in methods currently available after targeting β -tubulin and elongation factor 1 (eEF1). In agreement with previous studies, improved phylogenetic discrimination at the species and intraspecific level can be achieved via the analysis of genomic regions other than the ITS [16,25].

In this respect, O'Donnell and co-workers described a new strategy using β -tubulin and ITS marks to identify new strains in the *Fusarium* genus and explain that there are several factors relating to genetic, geographic, and ecological barriers which influence the chances of evolution genome in fungi, with useful references such as *A. tubingensis* CBS 1732, *A. carbonarius* CBS 120167, and comments on key assumptions implicit in methods currently available after targeting β -tubulin and elongation factor 1 (eEF1). In agreement with previous studies, improved phylogenetic discrimination at the species and intraspecific level can be achieved via the analysis of genomic regions other than the ITS [16,25]. We focus on the gene encoding elongation factor 1 (eEF-1) in order to investigate the genetic diversity amongst *Aspergillus* isolates. Our results suggested a full homology sequence

between *A. carbonarius* and *A. tubingensis*, which confirms our hypothesis. Moreover, the eEF-1 gene usually exhibits greater variability than β -tubulin gene sequences that are often used in taxonomic phylogenetics. Moreover, analysis of the eEF-1 sequence data has previously permitted the identification of intraspecific variation [26–28].

To date, the number of ongoing filamentous fungal genome sequencing projects is almost tenfold fewer than those of bacterial and archaeal genome projects. The fungi chosen for sequencing represent narrow kingdom diversity; most are pathogens or models. We advocate for an ambitious, forward-looking phylogenetic-based genome sequencing program designed to capture metabolic diversity within the fungal kingdom, thereby enhancing research into alternative bioenergy sources, bioremediation, and fungal–environment interactions.

5. Conclusions

The study aimed to unravel the phylogenetic relationships between two important fungal species, *A. carbonarius*, and *A. tubingensis*, via sequence analyses. After an in-depth analysis of genetic data and comparison with related species, several key conclusions can be drawn from this research:

Phylogenetic Placement: The sequence analyses provided valuable insights into the evolutionary relationships of *A. carbonarius* and *A. tubingensis* within the *Aspergillus* genus. These two species were found to be closely related to each other, forming a distinct clade within the phylogenetic tree.

Species Distinctiveness: Despite their close phylogenetic relationship, this study also highlighted the genetic differences that distinguish *A. carbonarius* and *A. tubingensis* as distinct species. These differences may include unique genetic markers or variations in key genes, contributing to their ecological and functional differences.

Evolutionary History: This research shed light on the evolutionary history of these species, suggesting a common ancestor and subsequent divergence. Understanding their evolutionary trajectory can help explain their ecological niches, adaptation strategies, and potential differences in mycotoxin production, which are crucial for agricultural and food safety considerations.

Taxonomic Clarification: This study contributes to the taxonomic clarification of these fungal species. Accurate species identification and classification are fundamental in various fields, including agriculture, mycology, and food safety, where *A. carbonarius* and *A. tubingensis* play significant roles.

Applied Implications: Knowledge of the phylogenetic relationships between these fungi can have practical applications in agriculture, industry, and biotechnology. It might help with the creation of plans for bioremediation, managing mycotoxin levels, and creating valuable metabolites.

In conclusion, this research provides valuable insights into the phylogenetic relationships, genetic distinctiveness, and evolutionary history of *A. carbonarius* and *A. tubingensis*. These findings not only contribute to our understanding of fungal evolution and taxonomy but also have practical implications for various fields, emphasizing the importance of continued research in fungal genomics and phylogenetics. Further investigations may delve deeper into the functional implications of these genetic differences and their ecological relevance.

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Institutional Review Board Statement: This statement acknowledges the absence of human subjects in the research and emphasizes the organization's commitment to ethical conduct across all research activities. It also encourages researchers to consider ethical considerations and seek guidance as needed, even in the absence of IRB oversight.

Informed Consent Statement: This statement ensures that participants are fully informed about the study before consenting to participate, thereby upholding ethical standards and protecting the rights and welfare of the participants.

Data Availability Statement: Data availability statements are available in section "MDPI Research Data Policies" at <https://www.mdpi.com/ethics>.

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Conflicts of Interest: The authors declare no conflicts of interest.

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