Unraveling Phylogenetic Relationships through *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 yearsof evolution. In this work, we report a clarification of the phylogenetic relationship between *A. carbonarius* and *A. tubingensis* by the screening of ITS-5.8RDNA, (niiA-niaD), ß-tubulin and eEF-1 genes. 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* exhibit a complete similarity (100%) for the three target genes: intergenic region of (niiA-niaD), ß-tubulin and eEF-1. Tree resulting from a bootstrap analysis indicate branch points when the significance is 98% minimum for each gene (ß-tubulin and eEF-1*)*. The phylogenetic analysis of aspergilli genome structure provided a quantitative evaluation of 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 evolutionary relationship between both species. At the same time, this finding can be one of the natural processes in the course of evolution of new taxa.

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

1. Introduction

The *Aspergillus* genus is widespread in nature [5,26], and many species of this genus have significant negative effects in agriculture, particularly the production of mycotoxins such as ochratoxin A [1,21,22]. The filamentous fungi *A. carbonarius* and *A. tubigensis* have been extensively employed in genetic research, and toxigenic potential study can be carried out on them [22]. The concern about these filamentous fungi in the vineyard has traditionallybeen linked to spoilage of grapes due to fungal growth. Nevertheless, these two saprobic fungican produce mycotoxins in grapes [15,25,28]. The phenotypic concept is the classic approach based on the morphological characteristics suggested that both species are far morphologically [8,11,13].

Partly, due to its economic importance, the genus *Aspergillus* has one of the better classifications among filamentous fungi [6,11]. Raper and Fennell in 1965 described 18 species groups within this genus based mainly on cultural and morphological features [25]. These were treated as sections belonging to 6 subgenera by Gams et al., 1985 [10]. Actually, phylogenetic studies of ribosomal RNA gene sequences led to the acceptance of

3 subgenera with a total of 15 sections [24]. In the present work, considerable attention is devoted to developing molecular tools to give a general overview of the taxonomic relationships among *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), ß-tubulin and eEF-1genes 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 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 (Calcbiochem, 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/μL.

2.2. PCR Amplification, Cloning and Sequencing

PCR amplification of ITS-5.8rDNA, intergenic region of (*niiA-niaD*), *ß-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, intergenic region of (*niiA-niaD*), *ß-tub* and *eF1* genes in a 50 μL reaction mixture containing 25 μL Hot Gold Star Mix, 1.2 μL of each primer (PN1-PN34), (Nit462F-Nit 873R), (*ßtub*F-*ßtub*R) and (e*EF1-1526*F- e*EF1*-1567R). The reactions were performedwith 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 *ß- tub*, and a last step at 72 °C for 1 min followed by a final incubation at 72 °C for 10 min. The amplification products were separated by horizontal electrophoresis on 2% agarose gel using Tris-Boron-EDTA buffer (TBE) 0.1X, for 1h at 100V. Fragment size was estimated using a suitable molecular marker (Smart DNA Ladder, Eurogentec, Belgium) and the PCR products were purified using PEG (Poly Ethylene Glycol) and purification spin-columns.

The clones amplified by PCR were subcloned in into *pGEMT* vector (Promega), and after heatshock, cloned in chemically competent Top10 one shot ® cells of *Escherichia coli* (Invitrogen, Karlsruhe, Germany). Cycle sequencing was conducted in a programmable "GeneAmp PCR 2700" thermal-cycler (AB-Applied Biosystem, CA, USA) using the primers PU-PR (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 sequencing reaction was carried out using the "CEQ™ 8000 Genetic Analysis System".

Table 1. xxx.

Primer Code	Primer Sequence (5′ to 3′)	Annealing Temperature (X) °C
PN1	AGTAAAAGTCGTAACAAGG	
PN34	TTGCCGCTTCACTCGCCGTT	55
PU	CGTTGTAAAACGACGGCCAGT	

PR	GTACCAGTATCGACAAAGGAG	52
Nit- 462F	GTTACGGAAGCAAAGAGGTA	
Nit -873R	GTTATTAGGGGCTATGGCAC	54
*ß- tub*F	CTCGAGCGTAGTAACGTCTAC	55
*ß- tub*R	AAACCCTGGAGGCAGTCGC	
eEF1-526F	GTCGTYGTYATYGGHCAYGT	58
eEF1-1567R	ACHGT RCC RATACCACC RAT CTT	

2.3. Sequence Alignment and Phylogenetic Analyses

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

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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). 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.

Regarding other black *Aspergillus* species, phylogenetic analysis of ITS sequence data indicates that at least several species belong to this section (*A. awamori*, *A. foetidus*, *A. tubingensis*, *A. niger* and *A. brasiliensis* are on a separate branch, while the uniseriate species *A. carbonarius* and *A. ibericus* are clustered together and defineda second clade. The last *Aspergillus* strain *A. flavus* was used as control (Figure 1).
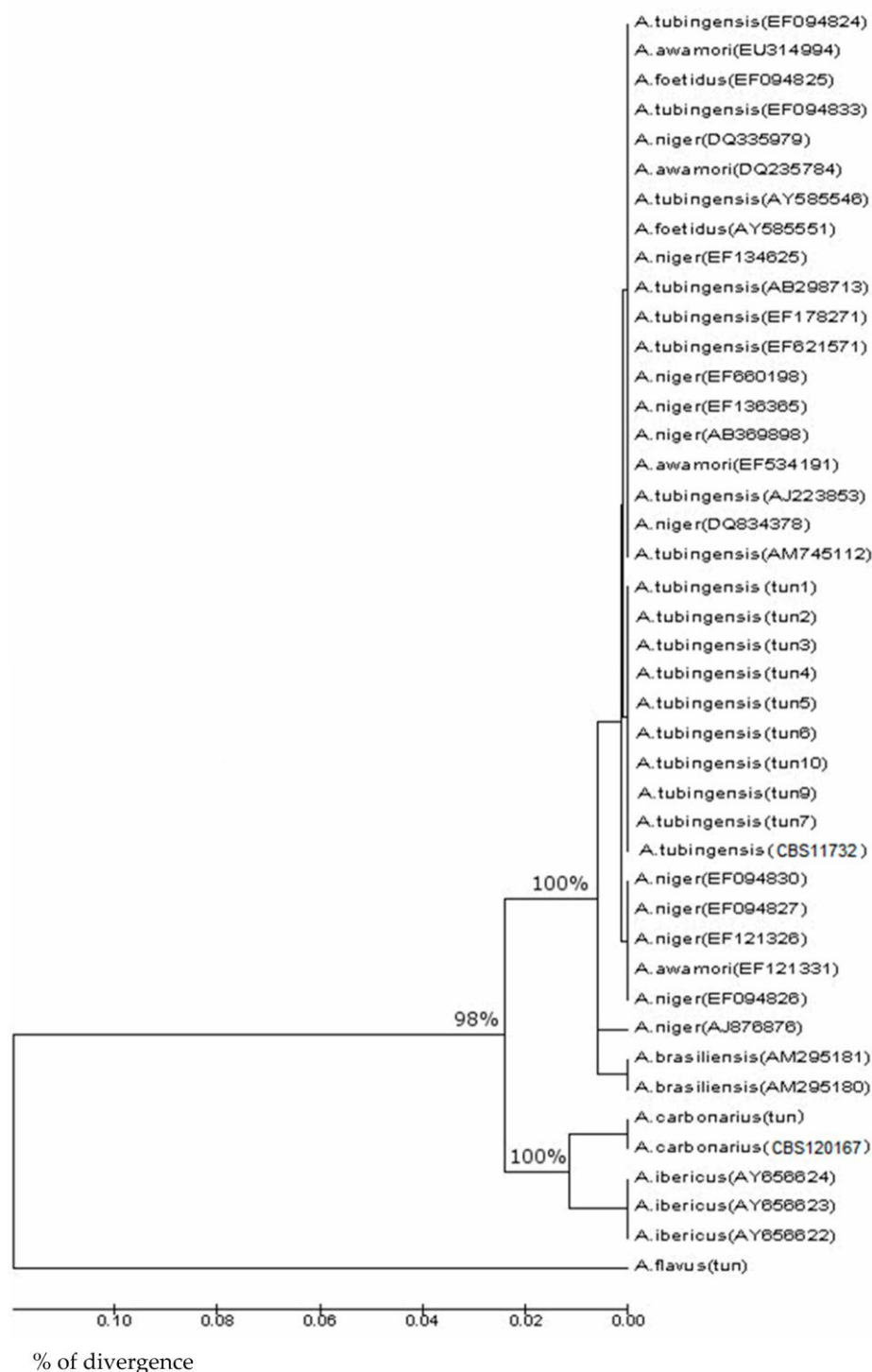
Figure 1. Neighbour-joining tree of ITS sequences of species assigned to *Aspergillus* section Nigri including *A. niger*, *A. tubingenis, A. awamori*, *A. foetidus*, *A.* and *A. brasiliensi*s clustered in the same branch. *A. carbonarius* and *A. ibericus* was clustered together and *A.flavus* was used as a positif control.

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

The intergenic portion of (niiA-niaD) gene was isolated within *A. carbonarius* and *A. tubingensis*. The nucleotide sequence suggests that *A. carbonarius* has twodistinct DNA fragments; one of these (400pb) was also amplified in *A. tubingensis* (Figure 2).The alignment and the comparison of the two sequences suggest a full homology between both

Aspergillus species. Following this result, a hypothesis has been expressed about the existence of phylogenetic relationship between both species. To confirm this hypothesis two genes have been target: ß-tubulin and eEF-1.
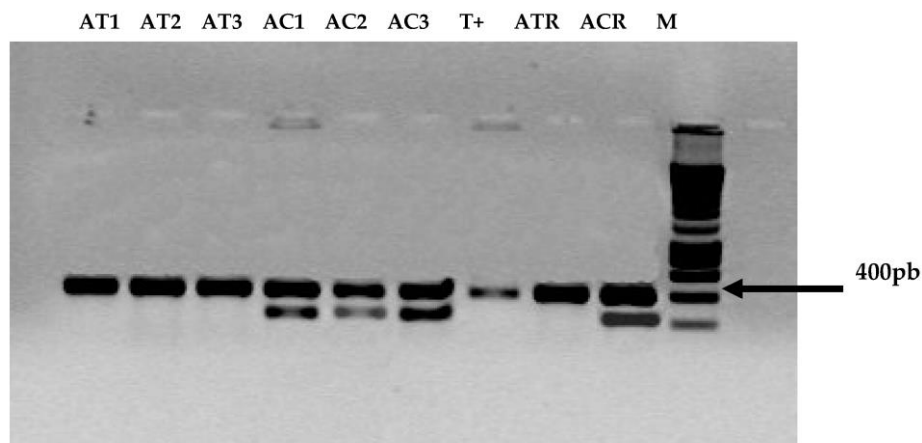

Figure 2. Results of the PCR used to target 400 pb of the intergenic portion of (*niiA-niaD*). From the left to the right (AT1-AT3): *A. tubingenis* isolates from grapes; AC1-AC3: *A. carbonarius* isolates; T+: positif control; ATR: *A. tubingenis* CBS11732 (reference strain); ACR: *A. carbonarius* CBS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

3.3. Screening and Phylogenetic Analysis of ß-tubulin Sequences

ß-tubulin gene was used to probe genomic DNA of *A. carbonarius* and *A. tubingensis*. Alignment of ß-tubulin genes isolated from *A. carbonarius*, *A. tubingensis*, *A. carbonarius* (CBS 120167), and *A. tubingensis* (CBS 11732) used as reference strain, gave evidence for a high degree of similarity (100%). In addition, the amino acid sequence deduced from the nucleotide sequence of ß-tubulin were used to develop a phylogenetic tree which indicate 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. tubigenis* (DQ902579), *A. tubigenis* 1–2, *A. tubingensis* (CBS 11732), *A. carbonarius* (CBS120167) and *A. carbonarius* 1–2 are clustered in group A; *A. foetidus* (DQ768454), *A. costaricaensis* (AY820014) are clustered in group B. The group C is composed of two branches with *A. brasiliensis* (DQ900611), *A. niger* (AY80001), (AY585535) and *A. lacticoffeatus* (AM419748). The last group is consists of *A. ibericus* (AM419748) and *A. sclerotioniger* (AY819996).

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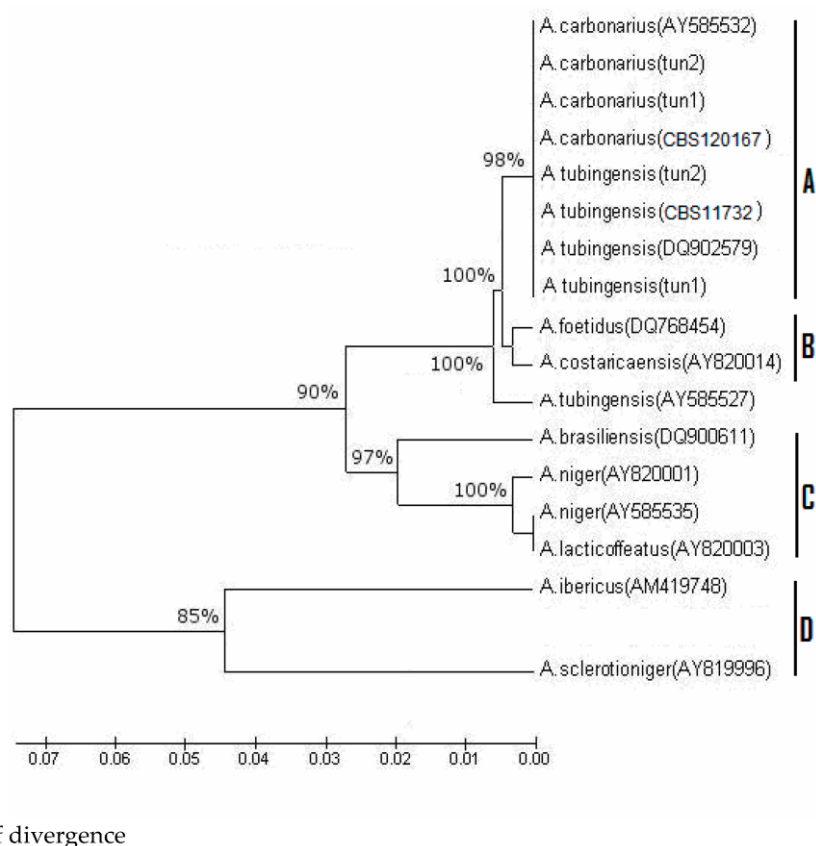
% of divergence

Figure 3. Phylogenetic tree based on amino acid sequences of *ß-tubulin* within *Ascomycetes*: *A. carbonarius* (AY585532), *A. tubingenis* (DQ902579), *A. tubingenis* 1–2, *A. tubingensis* (CBS 11732), *A. carbonarius* (CBS 120167) and *A. carbonarius* 1–2 are clustered in group (**A**); *A. foetidus* (DQ768454), *A. costaricaensis* (AY820014) are clustered in group (**B**) and related withgroup (**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 in *A. ibericus* (AM419748) and *A. sclerotioniger* (AY819996). Bootstrap percentages were calculated and ranged from 85% to 100%.

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. tubigensis* and *A. tubigensis* (CBS 11732).

Two distinct groups within the eEF-1 sequences were obtained for the 4 isolates of *Ascomycota* (*Aspergillus carbonarius*, *tubigensis*, *oryzae* and *Trichoderma gamsii*). Isolates within group A wereseparated 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).

The amino acid sequences were evident from both a visual inspection of the aligned sequences and the dendogram produced by analysis of the sequence data. DNA sequences from ß-tubulin and eEF-1 strongly supported the recognition of an evolution relationshipwithin the two species which is a favour of the hypothesis.
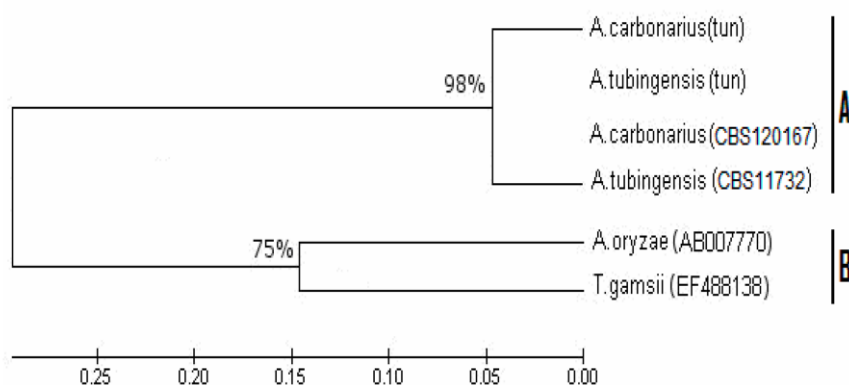
% of divergence

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, 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*).

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 recentyears the use of multiple gene analyses for phylogenetic studies has become more popular andseveral studies based on protein coding genes have been published including fungal taxa [4,9,23]. De Jong et al. (2001) showed that the analysis on multiple loci can provide more resolution and therefore more reliable phylogenetic hypotheses in fungal phylogeny. Phylogenetic analysis of sequences of the ribosomal ITS-5.8rDNA gene cluster and the ß- tubulin gene were found to be very useful for clarifying taxonomic relationships among toxigenic aspergilla [5].

In this report, ITS-5.8rDNA was used for *A. carbonarius* and *A. tubingensis* and analysis leads to two far distinguish species. In fact, previous work based on large subunit rDNA data has led to a widely accepted phylogeny of the aspergilli in which *A. tubigensis* and *A. niger* are more related to one another than *A. carbonarius* [7,26]. 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 present work, we report that 400pb of *(niiA-niaD)* gene target and subjected to a conserved sequence between *A. carbonarius* and *A. tubingenis*.

PCR assay for (niiA- niaD) conducted to a hypothesis of an evolution relationship. In disagreement with ourprevious work, intergenic region of (niiA-niaD) was used to distinguish between *A. niger* and *A. tubigenis* and two primers sets were designed for each species [6,22]. Here, our results based on sequence alignment, 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 [4,14], and (ii) in studies of complex species groups within protists, animals, fungi and plants [2,6,18,27].

In agreement with several studies, *β*-tubulin was usually used to detect evolutions in fungal species [9,14].

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

Thebasic 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 through the analysis of genomic regions other than the ITS [3,24].

In this respect, O'Donnell and co-workers described a new strategy using β-tubulin and ITS marks to indentify new strain in *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 through the analysis of genomic regions other than the ITS [3,24]. In this respect, O'Donnell and co-workers described a new strategy using β-tubulin and ITS marks to indentify new strain in *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 [16,20,23]. In order to study the genetic variability between *Aspergillus* isolates, we target the gene encoding elongation factor 1 (eEF-1). Our results suggested a full homology sequence between *A. carbonarius* and *A. tubingensis*, which confirm our hypothesis. Moreover, eEF-1 geneusually 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 [12,17,19].

To date, the number of ongoing filamentous fungal genome sequencing projects is almost tenfold fewer than those of bacterial and archaeal genome projects are. The fungi chosen for sequencing represent narrow kingdom diversity; most are pathogens or models. We advocate 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*, through 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, the 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: The 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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