Characterization of the translation elongation factor 1-α gene in a wide range of pathogenic Aspergillus species

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Abstract

Purpose. We aimed to evaluate the resolving power of the translation elongation factor (TEF)-1α gene for phylogenetic analysis of Aspergillus species.

Methodology. Sequences of 526 bp representing the coding region of the TEF-1α gene were used for the assessment of levels of intra- and inter-specific nucleotide polymorphism in 33 species of Aspergillus, including 57 reference, clinical and environmental strains.

Results. Analysis of TEF-1α sequences indicated a mean similarity of 92.6 % between the species, with inter-species diversity ranging from 0 to 70 nucleotides. The species with the closest resemblance were A. candidus/A. carneus, and A. flavus/A. oryzae/A. ochraceus, with 100 and 99.8 % identification, respectively. These species are phylogenetically very close and the TEF-1α gene appears not to have sufficient discriminatory power to differentiate them. Meanwhile, intra-species differences were found within strains of A. clavatus, A. clavatonicus, A. candidus, A. fumigatus, A. terreus, A. alliaceus, A. flavus, Eurotium amstelodami and E. chevalieri. The tree topology with strongly supported clades (≥70 % bootstrap values) was almost compatible with the phylogeny inferred from analysis of the DNA sequences of the beta tubulin gene (BT2). However, the backbone of the tree exhibited low bootstrap values, and inter-species correlations were not obvious in some clades; for example, tree topologies based on BT2 and TEF-1α genes were incompatible for some species, such as A. deflectus, A. janus and A. penicillioides.

Conclusion. The gene was not phylogenetically more informative than other known molecular markers. It will be necessary to test other genes or larger genomic regions to better understand the taxonomy of this important group of fungi.

INTRODUCTION

Aspergilli are biologically, economically and medically important fungi with vast ecological and metabolic diversity. They range from well-known pathogens such as Aspergillus flavus, which produces aflatoxin, through A. oryzae, which is involved in the industrial production of soy sauce, to A. niger, which is used for the production of acids and enzymes. Several species of Aspergillus may contaminate grains and other foods with toxic metabolites, threatening the health of humans and other animals, and may cause localized and systemic infections, especially in immunocompromised individuals, or cause different types of allergies [1–3].

A. fumigatus, is the predominant agent of invasive pulmonary aspergillosis (IPA) [2, 4], followed by A. flavus, A. terreus and A. niger; however, A. nidulans, A. ustus, A. alliaceus, A. lentulus, A. candidus, A. tubingensis and A. udagawae have also been implicated in IPA, although rarely so [4–7]. There are several reports of reduced antifungal drug susceptibility among Aspergillus species. For example, A. terreus isolates are resistant to amphotericin B, A. ustus isolates are refractory to azoles, and A. lentulus and A. alliaceus exhibit...
low *in vitro* susceptibility to a wide range of antifungals [5, 8–10]. Therefore, species delineation is essential for informing decisions on treatment, and for epidemiological and pathogenesis-related studies.

The traditional laboratory strategy for determining the identity/taxonomy of *Aspergillus* species is based on macroscopic colonial and microscopic morphological features [1, 4, 8, 11]. However, highly experienced microbiologists are required for such analyses. On the other hand, these methods cannot differentiate between cryptic and closely related species, the morphological features are largely unstable, and phenotypic analyses may require several weeks to complete [12].

Recent molecular data have supported relationships that were previously inferred from morphological and physiological characteristics [13]. Several different DNA loci, including non-coding regions and protein-coding genes, have been applied for sequence-based recognition of aspergilli, and extensive data are available in public databases. These loci include the mitochondrial cytochrome b gene [14], a putative aflatoxin pathway regulatory gene (*aflR*) [15], the actin gene [16], the DNA topoisomerase II gene (TOP2 [17]), various rRNA gene regions (D1/D2 in the 26S large subunit, 18S, and internal transcribed spacers 1 and 2, ITS1 and ITS2 [8, 18–20]), β-tubulin (BT2) and the calmodulin genes [21].

In spite of the shift of microbial identification formats into sequence-based approaches, no general consensus on which DNA target can be used for unambiguous species identification of *Aspergillus* has been achieved, especially for closely related species [4, 22].

The nucleotide sequence of the translation elongation factor 1-α (TEF-1α) gene, which encodes part of the protein translation machinery, has shown a high level of sequence polymorphism among related species of the genus *Fusarium* with a complex taxonomy, and has been considered as an alternative to rDNA for species identification [23]. Likewise, the gene is useful for developing robust phylogenetic inferences for other groups of pathogenic fungi, such as dermatophytes [24].

The usefulness of the TEF-1α gene in the systematics of aspergilli has not been fully elucidated. The majority of the TEF-1α sequences available in GenBank prior to this study were from species belonging to *Aspergillus* section *Nigri* [25, 26]. Therefore, the key goals of the present study were to characterize TEF-1α as a new genetic marker for evaluating intra- and inter-species variation, understand the relationships and species boundaries among important *Aspergillus* species, and determine whether sufficient variability exists for identification at the species level. We acquired DNA sequence information pertaining to TEF-1α regions for each of a wide range of common pathogenic and environmental species, and used both nucleotide and amino acid sequences for sequence alignments and pairwise sequence analyses. The preliminary data provided in the study could also be useful for improving the diagnostic detection and differentiation of *Aspergillus* in clinical, epidemiological, environmental and food science settings.

**METHODS**

*Aspergillus* strains and isolates

A total 57 strains, representing 15 sections and 33 different species of *Aspergillus*, including 49 reference strains and 8 clinical or environmental isolates, were used for the analysis of partial TEF-1α DNA sequences (Table 1). Reference strains were obtained from the Centraalbureau voor Schimmelcultures (CBS), Utrecht, the Netherlands, and Teikyo University Institute of Medical Mycology (TIMM), Tokyo, Japan. Clinical isolates were recovered from a variety of specimens, including nail, sputum, infected human tissues and broncho-alveolar lavage (BAL), and these were submitted to two medical mycology laboratories in Tehran, Iran. As the BT2 gene has been considered to be a good molecular marker for *Aspergillus* species identification [4], all of the samples were subjected to PCR and sequencing of partial BT2 gene using the fungal universal primers BT2a and BT2b [27], in order to identify the clinical and environmental strains and to reconfirm the reliability of the reference strains. The data were compared with reliable related sequences, deposited in GenBank using the BLAST algorithm, and the final species designations were in compliance with the names approved by the collections and/or reconfirmed by BLAST analysis of BT2 DNA sequences. The data were also used for phylogenetic analysis of *Aspergillus* strains and compared with a phylogenetic tree developed using TEF-1α DNA sequences.

**Primer design**

Initially, a previously reported primer pair [25] was used for PCR. The primers had been designed for amplification of the TEF-1α gene of *Aspergillus* species in the section *Nigri*, but they failed to successfully amplify all *Aspergillus* species included in our study. After sequencing some of the strains amplified by these primers and developing a multiple alignment of the resulting sequences with sequences previously deposited in GenBank, a new primer pair was manually designed and synthesized by Sigma–Aldrich (Sigma–Aldrich Co., USA) or Bioneer (South Korea) as follows: AspTef F (5’-AGC GTG GTA TCA CCA TCG-3’) as the forward primer and AspTef R (5’-GGA ACG TTC TTG AC G AGC TTG-3’) as the reverse primer.

**DNA extraction**

Fungi were grown on Sabouraud dextrose agar at 30 °C. An approximately 10–20 cubic mm piece of each fresh colony was added to a 1.5 ml tube containing 300 μl of distilled water and 250 μl of glass beads (0.5 mm in diameter). The tube was vortexed rigorously for about 5 min and centrifuged at 8000 r.p.m. for 1 min, and then 200 μl of the supernatant was subjected to DNA purification using an Exgene Tissue SV kit (GeneAll, Korea) according to the manufacturer’s instructions.
PCR amplification

A 2 µl aliquot of template DNA, 0.4 µM of each forward and reverse primer, 12.5 µl of premix (Ampliqon, Denmark) and enough water to make up a final volume of 25 µl were used for PCR amplification of the target genes, under the following conditions: initial denaturation for 5 min at 94 °C; 35 cycles of amplification, consisting of denaturation for 45 s at 94 °C; annealing for 1 min at 60 °C; and extension for 1 min at 72 °C, followed by an ultimate extension step at 72 °C for 5 min. The PCR products were electrophoresed on a 1.2 % agarose gel containing 0.5 µg ml⁻¹ ethidium bromide for about 1 h in TBE buffer (Tris 0.09 M, boric acid 0.09 M, EDTA 2 mM), and observed and photographed under ultraviolet irradiation.

Sequencing and sequence analysis

The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions, processed with an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA, USA), and

Table 1. Aspergillus strains used in this study and their sources and GenBank accession numbers

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain number (source, GenBank accession no.)</th>
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<tr>
<td>E. chevalieri</td>
<td>CBS 117329 (soil, KM921985), JCM 1568 (coffee beans, KM883007), CBS 121704 (ND, KT965678)</td>
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<td>E. amstelodami</td>
<td>CBS 119976 (indoor environment, Germany, KM921984), CBS 117312 (Manitoba, KP054367) JCM 1565 (ND, KP054371)</td>
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<td>E. intermedium</td>
<td>CBS 113.39 (unknown, KM921983)</td>
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<td>A. penicilloides</td>
<td>JCM 10256 [dried fish (ikan bilis) imported from Hong Kong, Australia, KM921972]</td>
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<td>A. mutans</td>
<td>CBS 122.56 (ND, KM883000)</td>
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<td>A. fumigatus</td>
<td>CBS 122886 (45-year-old-female, cutaneous ulcer after liver transplantation, China, KM921963), CBS 117202 (broncho-alveolar lavage, Netherlands, KM921964), CBS 109032 (patient, HIV-positive, invasive aspergillosis, Germany, KM921966), CBS 112389 (indoor environment, Germany, KM921962), CBS 118990 (broncho-alveolar lavage, France, KM921965), JCM 10253 (ND, KM921961)</td>
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<td>N. fischeri</td>
<td>CBS 681.77 (soil, Netherlands, KM921960)</td>
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<td>N. quadricincta</td>
<td>JCM 1855 (road board, KM921982)</td>
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<td>A. clavatonanasicus</td>
<td>CBS 122502 [isolated from a twig of T. mairei (Taxus mairei), China, KM921974], CBS 474.65 (fingernail lesion, Brazil, KM921978)</td>
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<td>A. clavatus</td>
<td>CBS 514.65 (dung, KP054380), JCM 10080 (stable manure, USA, KM226320)</td>
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<td>A. unguis</td>
<td>JCM 2256 (house cleaning wax, National Institute of Hygienic Sciences, Tokyo, Japan, KM882997)</td>
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<td>A. nidulans</td>
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<td>JCM 2729 (soil, New Jersey, USA, KM882991)</td>
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<td>A. sydowii</td>
<td>Ci 2419 (human nail, KP054369)</td>
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<td>A. versicolor</td>
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<td>A. janas var brevis</td>
<td>CBS 503.78 (maize-field soil, Colombia, KM921955)</td>
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<td>JCM 2731 (ND, KM921987)</td>
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<td>A. granulosus</td>
<td>CBS 119.58 (Soil, Texas, USA, KM882996)</td>
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<td>JCM 10114 (sake koji, KM921969)</td>
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<td>P. alliaceus</td>
<td>CBS 511.69 (soil, Izmir, Turkey, KP054373), CBS 612.78 (Buenos Aires, Argentina, KM226322)</td>
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<td>A. tubingenensis</td>
<td>CBS 122719 (ND, KM883006), CBS 107.55 (ND, KM882994)</td>
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<td>CBS 266.81 (wheat grain, India, KM921954), CBS 225.80 (human nail, Netherlands, KM883003), Ci 2409 (human nail, KM882995), JCM 10250 (ND, KM883002), Eni 23 (spice, KM883004)</td>
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<td>A. varians</td>
<td>JCM 2760 (ND, KT965679)</td>
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ND, not determined, Ci, clinical isolate. Eni, environmental isolate.
individually sequenced bidirectionally, using the same primers as for the primary PCR, via an automated DNA sequencer (ABI Prism 3730 Genetic Analyzer, Applied Biosystems). The sequences were edited with Geneious software (www.geneious.com), and consensus sequences were used for further analysis. The pairwise comparisons, multiple alignment, levels of sequence identity and sequence difference counts were calculated using BioEdit (www.mbio.ncsu.edu/bioedit/bioedit.html) or Geneious software.

For tree construction, consensus sequences were imported into MEGA6 software [28], a phylogenetic tree was constructed and the tree topology was evaluated manually for congruence of species-rank clades using already described conditions [24].

**Nucleotide sequence accession numbers**

All the *Aspergillus* TEF-1α sequences obtained in this study and their corresponding amino acid sequences were deposited in GenBank and assigned the accession numbers listed in Table 1.

**RESULTS**

To evaluate the applicability of TEF-1α DNA sequences for the differentiation and phylogenetic study of *Aspergillus* species, partial TEF-1α genes were examined for 57 strains of *Aspergillus* (Table 1). Using the primer pair AspTef F and AspTef R, we were able to successfully amplify a single PCR fragment for all of the strains included in the study, and this was followed by analysis of the DNA sequences and a nucleotide-based phylogenetic analysis. Multiple alignment of sequences pertaining to TEF-1α indicated a mean similarity of 92.6% between the species (Fig. 1). The size of this fragment, excluding the uncertain first and last parts of the sequence, was identical across all of the strains, namely 526 nucleotides (nt), and the region was seen not to contain any introns.

A sequence difference count matrix between these strains showed differences ranging from 0 to 71 nucleotides (data not shown), with the largest distance being observed between *A. varians* and *A. penicillioides*. Meanwhile, intra-species differences were found within strains of *A. clavatus*, *A. clavatonanicus*, *A. candidus*, *A. fumigatus*, *A. terreus*, *A. alliaceus*, *A. flavus*, *Eurotium amstelodami* and *E. chevalieri*.

Fig. 2 shows the TEF-1α taxonomic naming framework, as computed using MEGA6 software. A TEF-1α sequence representing *Fusarium oxysporum* was used as an outgroup. The topology was similar to that observed in the BT2 tree gene (Fig. 3), and most species, in particular closely related taxa, clustered in similar, strongly supported clades (>70% bootstrap values). However, the backbone of the tree exhibited low bootstrap values, and inter-species correlations were not obvious in some clades. Sequence variation between the *Aspergillus* strains led to the identification of four clusters with different sections of species.

The species of the section *Flavi* were divided into two sub-clades: sub-clade 1, with typical strains of *A. flavus*, *A. tamari*, *A. oryzae* and *A. ochraceus*, supported by a bootstrap value of 97%, and sub-clade 2, including *A. avenaceus* and *A. alliaceus* as a separate group with a bootstrap value of 100%. Phylogenetic analysis of the respective BT2 genes saw all the mentioned species segregate into one clade with a bootstrap value of 100% (Fig. 3).

The sequences of two closely related species, *A. flavus* and *A. oryzae*, differed from each other by only a single nucleotide (position 360), with the ’C’ in *A. flavus* being replaced by a ’T’ in *A. oryzae*. In the tree constructed based on the TEF-1α gene, strains of *Aspergillus* section *Nigri* were located next to the *Flavi* section. The sequence difference count matrix based on nt pairwise comparison of the BT2 gene showed 81.8% identification for two closely related species, *A. niger* and *A. tubingensis*, and 97.9% identification for the aforementioned species was observed based on TEF-1α.

Phylogenetic analysis of the TEF-1α genes revealed a cluster consisting of the *Aspergillus* subgenus *Fumigati*, including section *Clavati* and section *Fumigati*. Molecular data revealed that the *Aspergillus* section *Fumigati* consists of three species: *A. fumigatus*, *N. fischeri* (*A. fischeri*) and *N. quadricincta* (*A. quadricinctus*). Strains of *A. clavatus*, *A. clavatonanicus* and *A. nutans* were found to be closely related, forming a distinct sub-clade next to the *Fumigati* section.

Phylogenetic analysis of the TEF-1α genes revealed a cluster consisting of *Aspergillus* section *Candidi*, *Terrei* and *Flavipes*, in which the members were classified into two groups (Fig. 2).

Parsimony analysis of the DNA sequence data indicated that species of *Aspergillus* section *Versicolores* (*A. versicolor*, *A. sydowii*, *A. varians* and *A. unguis*), section *Usti* (*A. ustus*, *A. granulosus* and *A. deflectus*) and section *Nidulantes* (*A. nidulans* and *E. rugulosa* (*A. rugulosus*)) form their own distinct cluster. Likewise, phylogenetic analysis of BT2 genes indicated that the three sections *Nidulantes*, *Usti* and *Versicolores* formed a separate cluster. *A. ustus* and *A. granulosus*, supported by a low bootstrap value (63%), formed a branch closely related to *Aspergillus* section *Versicolores*. The tree constructed based on BT2 DNA sequences indicated that *A. deflectus* belongs to the section *Usti*, while TEF-1α gene analysis revealed that *A. deflectus* stands on a lineage that is closely related to section *Nidulantes*.

In the TEF-1α gene analysis, an *A. junus* strain was found to form a separate lineage closely related to the clade consisting of sections *Nidulantes*, *Usti* and *Versicolores*, and excluded from *Aspergillus* section *Flavipes*. Further, tree topology and bootstrap analysis indicated that *A. penicillioides* is more closely related to *E. amstelodami*, *E. intermediate* and *E. chevalieri*, which are all members of *Aspergillus* section *Aspergillus*, while the tree topology based...
Fig. 1. Multiple sequence alignment of partial TEF-1α gene sequences from 33 representative Aspergillus species. A dot indicates an identical nucleotide with respect to the top sequence and a dash indicates an insertion/deletion (indel) event.
on BT2 genes placed the mentioned species next to section Nigri.

DISCUSSION

Species of the genus Aspergillus are widely dispersed everywhere in the environment [29]. The genus is divided into seven subgenera, which are subdivided into a number of sections, with each section containing a few to several closely related species [9].

Although A. fumigatus is the most common etiologic cause of invasive aspergillosis, the role of non-fumigatus Aspergillus species is being increasingly recognized [29, 30]. Recently, rare species isolated from invasive aspergillosis have been reported, including A. ustus, A. alliaceus, A. lentulus [31] and A. udagawae [32].

The taxonomy and evolutionary relationships of Aspergillus species are based on the morphological characteristics, extrolite data and partial DNA sequences of different genetic targets [15]. However, lack of pigment or poor sporulation, inter-specific similarities and intra-specific variability, and variation in growth requirements for some isolates may influence the outcome of identification [33, 34]. Therefore, molecular methods and algorithms are
necessary for distinguishing and/or (re-)classifying similar and complex *Aspergillus* taxa, as well as for the discovery of new species.

An international *Aspergillus* working group has recommended the use of molecular identification based on the ITS region for subgenus/section-level identification [4]. However, comparative sequence-based identification using the ITS1 region does not always enable discrimination between closely related species [35, 36], which might be due to inadequate sequence variability or issues with the reliability of the ITS sequences deposited in reference databases (GenBank, EMBL, DDBJ) [37]. Some housekeeping genes, such as BT2, calmodulin and rodlet A, have been confirmed as good genetic indicators for identifying species within different sections, such as *Fumigati*, *Usti*, *Nigri* and *Terrei* [16, 38, 39], but they are not able to differentiate, for example, species that are phylogenetically close to *A. parasiticus* [21], and therefore sequence analyses of other loci are required for accurate identification.

Owing to polymorphism and DNA sequence length differences in introns, intron-rich protein-coding genes such as *β*-tubulin, calmodulin, actin and TEF-1α are recommended for the discrimination of fungal species [24, 40, 41]. Hence, the identical size (526 nt) and high homology (92.6%) observed among all the strains reflected the lack of introns in the TEF-1α gene region studied here.

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**Fig. 2.** Phylogenetic analysis of 33 representative *Aspergillus* species based on analysis of TEF-1α gene sequences. The evolutionary history was inferred using the neighbor-joining (NJ) method, based on the Tamura–Nei model.
Phylogenetic analysis (Fig. 2) of the TEF-1α genes showed a clade consisting of A. flavus, A. oryzae, A. ochraceus and A. tamari (Flavi section), supported by a bootstrap value of 97 %, next to the members of A. avenaceus and A. alliaceus, as a separate group with a bootstrap value of 100 %. However, phylogenetic analysis of BT2 genes placed all the mentioned species, except A. ochraceus, next to A. janus, in one clade, with a bootstrap value of 100 % (Fig. 3).

Based on morphological features, Thom and Raper [42] and Kozakiewicz [43] assigned the A. alliaceus species to the Aspergillus section Wentii, but later, Varga et al. [7, 44] and Frisvad and Samson [45] found that A. lanosus and anamorphs of A. alliaceus are closely related to Aspergillus section Flavi.

Varga et al. [15] provided an overview of Aspergillus section Flavi, using morphological features, extrolite data, and analysis of the ITS region and partial calmodulin and β-tubulin genes to examine the evolutionary relationships within this section. They found that Aspergillus section Flavi includes 22 species, which can be grouped into 7 clades of A. flavus, A. avenaceus, A. alliaceus, A. tamari, A. nomius, A. togoensis and A. leporis. Kozakiewicz [43] suggested that A. avenaceus might be related to A. alliaceus, based on morphological features; however, sequence data using partial calmodulin, β-tubulin and ITS sequences to investigate the Flavi section did not support this view and assigned this species to a separate clade, distant from A. alliaceus strains [15].

Pairwise comparison of the DNA sequences of both the TEF-1α and BT2 genes in this study showed a single SNP difference between A. flavus and A. oryzae. Regarding the evolutionary origins of A. oryzae and A. flavus, based on the region neighboring the cyclopiazonic acid biosynthesis gene cluster, Chang et al. [46] suggested that A. oryzae most likely descended from an ancestor that was the
ancestor of *A. minisclerotigenes* or *A. parvisclerotigenus*, producing both B- and G-type aflatoxins, while *A. flavus* descended from an ancestor of *A. parasiticus*. Although several lines of evidence show that *A. oryzae* is a morphological variant of *A. flavus*, it was suggested that these taxa should be retained as separate species because of the regular confusion that conspecificity might generate in the food industry [15, 47].

Differentiation of some species of black aspergilli, such as *A. niger* and *A. tubingensis*, which are common in both clinical and environmental settings, remain difficult [48]. The taxa can be differentiated by DNA sequences reflecting the cytochrome b [49], ITS [50] and β-tubulin [51] genes. In this study, a sequence difference count matrix based on nt pairwise comparison of the BT2 gene with a similarity of 81.8 % provided evidence that this locus is more useful than TEF-1α (97.9 % similarity) for species discrimination of these two closely related species.

The phylogenetic tree of the TEF-1α genes revealed a cluster consisting of *Fumigati* and *Clavati* sections. Closely related species, *A. fumigatus*, *A. fischeri* and *A. quadricinecta* (section *Fumigati*), formed well-supported clades in the TEF-1α and BT2 gene analyses, with bootstrap values of 98 and 100 %, respectively. The phylogenetic tree derived from TEF-1α indicated that *A. clavatus*, *A. clavatonanicus* and *A. nutans* (section *Clavati*) form a sister group with section *Fumigati*, with a bootstrap value of 76 %, and the phylogenetic analysis of partial DNA sequences of BT2 genes provided a bootstrap value of 100 %.

Through the alignment of TEF-1α DNA sequences, six strains of *A. fumigatus* were grouped in a cluster with 96 % bootstrap support, with a maximum of one nt difference from each other, and clearly separated from *N. fischeri* and *A. quadricinecta*. This finding was in concordance with observations by Hong et al. [16] and Wang et al. [14], who used partial β-tubulin, calmodulin, actin and mitochondrial cytochrome b gene analysis of *A. fumigatus* and concluded that the intra-specific genetic variability within *A. fumigatus* was low, whereas intra-specific morphological diversity within the species was high.

TEF-1α grouped *A. terreus*, *A. candidus*, *A. carneus*, *A. flaviipes* and *Fennelia nivea* (*A. neoniveus*) as a separate clade next to the *A. chevalieri* clade consisting of *E. amstelodami*, *E. chevalieri* and *E. intermedium* species (Fig. 2). Likewise, Varga et al. [52], using ITS regions, showed the presence of three main clades, which included *A. terreus*, *A. carneus* and *A. niveus* within the section *Terrei*. By contrast, the applicability of comparative sequence analysis of the D1 and D2 regions proved to be of limited utility for studying relationships within the section *Terrei*, while ITS regions were useful owing to more nucleotide diversity in this region [52]. Given the fact that numerous cryptic species may be present within the section *Terrei*, it can be concluded that it warrants further detailed molecular analysis, with the inclusion of more loci and isolates. The present study showed that the intra-species nt differences enabled discrimination between two and three distinct TEF-1α genotypes within strains of *A. terreus* and *A. candidus*, respectively, which could be useful for genotyping purposes.

In the present study, the TEF-1α and BT2 DNA sequences were identical for *A. candidus* and *A. carneus* strains, while in silico analysis of BT2 DNA sequences from GenBank showed differences of 180 nt between these species. It is possible that contamination with DNA could have occurred during sample collection or sample processing in the laboratory. Therefore, due to the low number of tested species, further studies on more isolates are required to confirm the results. Three sections, *Nidulantes*, *Usti* and *Versicolores*, were located in three monophyletic groups in a separate cluster in the TEF-1α and BT2 phylogenetic trees. Most of the species belonging to section *Usti* have been placed in sections *Versicolores* or *Nidulantes* [21]. Based on sequence analysis of partial 28S rRNA genes, Peterson [53] transferred most species of section *Usti* to section *Nidulantes*, with *A. ustus* and *A. granulosus* forming a well-supported branch closely related to *A. versicolor*. Balajee et al. [4], through analyses of D1–D2 sequences deposited in GenBank, showed two clades in section *Usti*; one clade consisted of *A. ustus*, *A. punicicus* and *A. ustus* var. *laevis*, and the other clade included *A. granulosus* and *A. pseudodefectus*. However, they concluded that the D1–D2 region does not have sufficient sequence variation for species delimitation. Further, analysis of a part of the 28S rRNA gene indicated that *A. deflectus* is positioned on another branch related to *A. elongatus* and *A. lucknowensis* in the *A. versicolor* group [53]. Although supported by a low bootstrap value, the phylogenetic tree developed based on our BT2 DNA sequences indicated that *A. deflectus* belongs to the section *Usti*, while analysis based on the TEF-1α gene revealed that *A. deflectus* stands on a separate branch closely related to *A. rugulosus* and *A. nidulans*. TEF-1α and BT2 DNA sequence analysis indicated that section *Versicolores* only includes *A. versicolor* and *A. sydowii*, forming a monophyletic group supported by 97 and 87 %-bootstrap values, respectively. A phylogenetic tree reflecting DNA sequence data from four loci, including calmodulin, RNA polymerase 2, DNA replication licensing factor Mcm7 and pre-rRNA processing protein Tsrl, indicated that the section *Versicolores* contains three subclades: *A. versicolor*, *A. sydowii* and *A. subversicolor*, where *A. sydowii* and *A. versicolor* were each identified as independent lineages [54].

The present study showed that three isolates of *E. amstelodami*, three isolates of *E. chevalieri* and one isolate of *E. intermedium* formed the *E. chevalieri* clade, with bootstrap values of 66 and 100 % in TEF-1α and BT2 phylogenetic trees, respectively. This finding is in concordance with data published by Stephen et al., who identified *E. chevalieri* (*A. chevalieri*) as a sister species of *E. amstelodami* (*A. amstelodami*) and *E. intermedium* (*A. intermedius*), based on phylogenies inferred from combined BT2, calmodulin and RNA polymerase II data [21].
In conclusion, the present study was carried out to assess the relationships and boundaries between *Aspergillus* species using a new genetic marker, the TEF-1α gene. In general, the phylogenetic inferences based on TEF-1α and BT2 were similar, with only very small discrepancies being identified between the two phylogenies. *A. penicilloides* was more closely related to the *A. chevalieri* clade (members of section Aspergillus) according to TEF-1α gene analysis, while the tree topology based on BT2 gene analysis showed the species as being next to the section *Nigri*. On the other hand, the phylogenetic analysis based on TEF-1α genes placed *A. ochraceus* in the Flavi section. However, based on our BT2 phylogenetic tree, *A. ochraceus* and *A. janus* formed a monophyletic group, which could be described as a separate section. So, the gene was not phylogenetically more informative than other known molecular markers, and it will be necessary to test other genes or larger genomic regions to better understand the taxonomy of this important group of fungi.

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Conflicts of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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