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Phylogenetic analysis of HSP70 gene of *Aspergillus fumigatus* reveals conservation intra-species and divergence inter-species

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Abstract:

Aspergillus fumigatus is a saprophyte fungus, widely spread in a variety of ecological niches and the most prevalent aspergilli responsible for human and animal invasive aspergillosis. The first step to develop novel and efficient therapies is the identification and understanding of the key tolerance and virulence factors of *pathogens*. The main focus of the present study is to perform the similarity, conservation and phylogenetic analysis of heat shock protein 70 (HSP70) gene of the fungus *A. fumigatus*. Therefore, DNA sequence of HSP70 gene was obtained from a native airborne *A. fumigatus*. Similarity, divergence and conservation of HSP70 gene sequence were assayed and compared to other strains and fungal species using nucleotide Basic Local Alignment Search Tool (BLAST), construction of phylogenetic tree and online analysis tools. The results revealed that the most similar aspergilli to the examined *A. fumigatus* strain were *A. fumigatus* non-native strains, *Aspergillus clavatus*, *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus nidulans*. Among other fungal genera, *Penicillium* species were the most similar fungal genus to *A. fumigatus.* Moreover, the alignment results revealed high levels of similarity and conservation of native *A. fumigatus* HSP70 gene sequence with yeasts stress-Seventy subfamily A (SSA) gene sequence from HSP70 family of genes and lower similarities with other genes in the family (Ssb, Ssc, HscA, Kar2 and bipA). *Penicillium* spp. were the most similar fungi to *Aspergillus* species by phylogenetic analysis of HSP70 sequence. Other molds, yeasts and yeast-like fungi were placed in more distanced clades. In general, fungal HSP70 sequence analysis could direct us to make a better understanding of thermo-tolerance in different fungal species.

Keywords: *Aspergillus fumigatus*, heat shock protein 70, phylogenetic tree, phylogeny, diversity.

Introduction

Aspergillus fumigatus is an opportunistic filamentous fungal pathogen which is ubiquitous in the environment (Latgé, 1999). It spreads considerable concentrations of asexual conidia (spores) into the air to propagate itself (Goodley et al., 1994;

O'Gorman, 2011). The inhalation of conidia is the main route of infection among immunocompromised patients (Dagenais and Keller, 2009). Moreover, in an impaired immune system, the conidia could germinate into invasive hyphae, which can cause extensive damage to lungs and other organs. In immunocompromised patients, the prognosis for invasive aspergillosis (IA) is poor (Lin et al., 2001). Spores of *A. fumigatus* are not widespread in the atmosphere compared to the conidia of some nonpathogenic molds (Hospenthal et al., 1998). Thus, it is hypothetical that thermo tolerance attitude of *A. fumigatus* plays an important role in survival of the organism through the host body (Latgé, 1999). *A. fumigatus* has the ability of growing rapidly at 37°C. It can also tolerate higher temperatures (up to 60°C) (Beffa et al., 1998; Holder and Lewis, 2003). One of the most important genes involved in high temperature tolerance is heat shock protein (HSP) gene, controlling the production of HSPs (Burnie et al., 2006). HSPs are normally present in eukaryotic and prokaryotic cells (Zhang et al., 1998) and their expression levels increase under stress conditions (Jolly and Morimoto, 2000). It has been shown that the expression of HSP70 prepares a condition for fungi to adapt to new environmental situations (Allendoerfer et al., 1996).

Comparative evolutionary analyses are the fundamental tools in biology, focusing typically on the conserved features; these analyses have been employed in examining interspecies differences. The amount of change throughout the sequences reflects the evolutionary relatedness of the organisms (Yang and Bielawski, 2000).

The aim of the present study is to perform the comparative analysis, phylogenetic inferring, and understand the similarities and differences of fungal HSP70 sequences between our native *A. fumigatus* strain and other *Aspergillus* species,which were not examined under the earlier independent study. Also, this study will focus on the analysis and interpretation of molecular diversity of HSP70 in other fungal genera, such as *Penicillium* species, *Dermatophytes* and *Candida* species.

Materials and Methods

Strains and growth conditions

An *A. fumigatus* wild-type strain originally isolated from air in Tehran, Iran, was obtained from fungal collection of Mycology Research Center, Faculty of Veterinary Medicine, University of Tehran, Iran. The fungus was cultured in a slant culture using the Sabouraud glucose agar and incubated at 25°C. Conidia were harvested from 7-day-old cultures by pouring a sterile 0.01% aqueous solution of Tween 80 on slant cultures surface and thereafter in order to facilitate the release of conidia, the culture surface was scraped with a bent glass rod. Number of conidia in the suspension was adjusted to approximately 10^8 conidia/ml. The conidia suspension was inoculated in Sabouraud dextrose broth and then incubated at 42°C for 5 days.

Preparation of chromosomal DNA

Genomic DNA from native *A. fumigatus* was extracted using the DNeasy Plant Mini Kit, QIAGEN, Germany, by means of the process recommended by the manufacturers.

Amplification protocol

Gene-specific primers of HSP70 were designed using online primer design software (GenScript Corp., Piscataway, NJ,

USA). The HSP70 gene sequence of native *A. fumigatus* was amplified by using forward:

5´-TGACCATTGAGGAGGGTATCTTC-3´ and reverse: 5´-AAGAGGTGTCACCAGAC AGGATAG-3´ primers. Polymerase chain reaction (PCR) assays were carried out in 25 µl reaction volume containing 12.5 µl of 2X PCR Master Kit (SinaClon BioScience, Iran), 1 µl from each of the forward and reverse primers (10 pmol), 5.5 µl diethylpyrocarbonate (DEPC) water (SinaClon BioScience, Iran) and 3 µl of DNA template (50 ng). The PCR program was started at 95°C for 4 min, followed by 38 cycles, including denaturation at 94°C for 1 min, annealing at 59°C for 1 min and extension at 72°C for 90 s, with a final extension step of 72°C for 5 min.

PCR product purification

By using a gel extraction kit (Thermo Fisher Scientific, USA), the amplified PCR product was purified from the agarose gel. The PCR products were analyzed in 1.5% agarose gel for 90 min at 80 V in 1X tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer and then stained with ethidium bromide (25 µg/ml) for 15 min (SinaClon BioScience) (Figure 1).Also, a 100 bp DNA ladder was loaded (Thermo Fisher Scientific, USA).

Sequencing of PCR product

The purified PCR product was sequenced, using ABI PRISM®BigDye™ terminator cycle sequencing kits (Macrogen Inc., South Korea). The samples were analyzed using an ABI PRISM® 3730XL automated sequencer. It is necessary to mention that sequencing was performed in triplicates. Two directional sequencing was used to read the sequences.

Sequence registration

Bioedit version 7.2.5 was used to edit sequence data. The HSP70 gene sequence of Iranian native *A. fumigatus* was then registered by Sequin online software at the National Center for Biotechnology Information (NCBI). This sequence was approved by GenBank with accession number: KC981070.

Basic Local Alignment Search Tool (BLAST) and alignment of sequences

Our HSP70 gene sequence was directed to nucleotide BLAST and was searched for its similarity using the nucleotide database of GenBank. Also, for further alignment analysis, nucleotide sequences of the HSP70 gene from different *A. fumigatus* strains and other fungi were retrieved from GenBank database. Reference sequences (RefSeq) were also analyzed by internal BLAST feature in *Aspergillus* Genome Database (AspGD) (Arnaud et al., 2010; Cerqueira et al., 2014). The obtained sequences were aligned using Clustal Omega at European Bioinformatics Institute (EMBL-EBI) website (Chenna et al., 2003) and trimmed in CLC Sequence viewer software version 7.0.2. In this step of the study, very short sequences and areas with ambiguous alignment or containing poly-N stretches were excluded from the analyses.

Similarity and conservation analysis

Multiple sequence alignment file that resulted from prior Clustal Omega analysis and trimming step consisting of 99 sequences of important fungal species was also employed as input file to perform the conservation analysis using ConSurf online

tool (Ashkenazy et al., 2010) and similarity analysis using YASS online tool (Noe and Kucherov, 2005). Native *A. fumigatus* 389 bp sequence (after trimming ambiguous areas) was introduced as reference sequence to ConSurf program. All the settings of ConSurf and YASS tools remained as their default values.

Constructing the phylogenetic tree

Sequences of the native and non-native *A. fumigatus* HSP70 gene, other *Aspergillus* species and other fungi were retrieved from GenBank database to make a phylogenetic analysis. The sequences were compared using CLC sequence viewer version 7.0.2. The data set were exported to MEGA 6.0.6 software packages (Tamura et al., 2011) to construct the phylogenic tree. To keep high the robustness and confidence of the tree, bootstrap was set on 100. Aligned sequences were used to perform the phylogenetic analysis. *Escherichia coli* HSP70 sequence was used as out-group in phylogenetic analysis. In addition, the total mean distances were calculated. Tree was constructed using neighbor-joining (NJ) algorithm under the global gap removal option and Kimura's two-parameter substitution model (Kimura, 1980). The percentage of replicated trees in which the associated taxa clustered together in a bootstrap test (100 replicates) is shown next to the branches (Felsenstein, 1985).

Results

Gel electrophoresis

As shown in Figure 1, the planned PCR process could amplify the desirable fragment of HSP70 gene of *A. fumigatus* (524 bp).

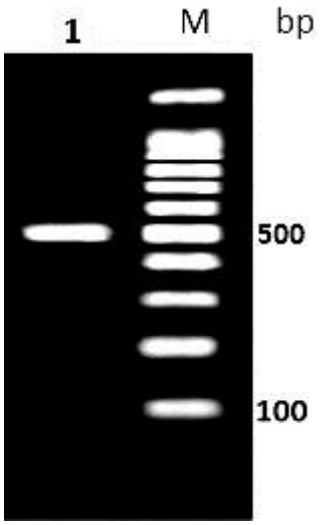

Fig. 1. Agarose gel electrophoresis of PCR products from native *A. fumigatus*; lane M: 100 bp DNA Ladder, lane 1: 524 bp PCR product of *A. fumigatus* genomic DNA using HSP70 forward and reverse primer set.

Similarity analysis

BLAST results demonstrated that *Aspergillus niger, Aspergillus clavatus, Aspergillus terreus,* and *Aspergillus nidlulans* have 94, 94, 93, and 88% similarity with our native sequence, respectively. Alongside, our sequence showed a rate of 100% similarity when compared to four other *A. fumigatus* HSP70 sequences retrieved from the GenBank. According to the results of *Aspergillus* intra-species similarity analysis of HSP70 gene sequences, the closest strains to native *A. fumigatus* were the other strains of *A. fumigatus* and other *Aspergillus* spp., such as *A. niger, A. clavatus, A. terreus* and *A.nidulans,* respectively. These results showed high comparability and conservation among HSP70 genes in *A. fumigatus* strains. BLAST analysis of HSP70 gene in different fungal species compared to native *A. fumigatus* strain showed that the most similar non-*Aspergillus* fungal species to the native *A. fumigatus* were *Penicillium* spp., *Dermatophytes* and *Alternaria alternata,* respectively. BLAST analysis in AspGD and NCBI's BLAST revealed that our native

sequence was the most similar orthologous to yeasts Stress-Seventy subfamily A2 (SSA2), SSA4 and HSP70 gene sequences belonging to HSP70 genes family. The sequence exhibits the highest similarity to SSA2 gene of yeasts, including *Saccharomyces cerevisiae*, *Candida albicans* and *Schizosaccharomyces pombe* and to HSP70-1 gene in *Neurospora crassa*. Moreover, using BLAST to search similarity among fungal species resulted in high identity and query coverage scores indicating high similarity of our sequence to SSA4 gene sequence, belonging to HSP70 gene series. Other genes that participated in HSP70 family, such as SSB, SSC, bipA, Kar2 (SSD) and HscA showed lower similarity measures to our native sequence. Results of multiple sequence alignment (MSA) of *A. fumigatus* and other fungi have specified the conserved and variable regions (Fig. 2) stretched in different parts of protein. Alignment revealed high similarity and conservation of HSP70 gene in different fungal species and genera. Although, there were remarkable differences in HSP70 gene sequences between *Candida* spp. and other fungal species.

Conservation analysis

Results of conservation analysis using ConSurf online tool revealed high conservation ratio of HSP70 sequence of native *A. fumigates* when compared with other fungal HSP70 sequences (Fig. 2).The results also demonstrated that 239 nucleotide locus (61.4%) of 389 analyzed locus were categorized as the conserved positions (data not shown). The conservation scale in ConSurf results was defined as a score from 1 to 9 which is calculated based on either an empirical Bayesian method or a maximum likelihood (ML) method. Each nucleotide locus with conservation score higher than 5 were considered as conserved locus.

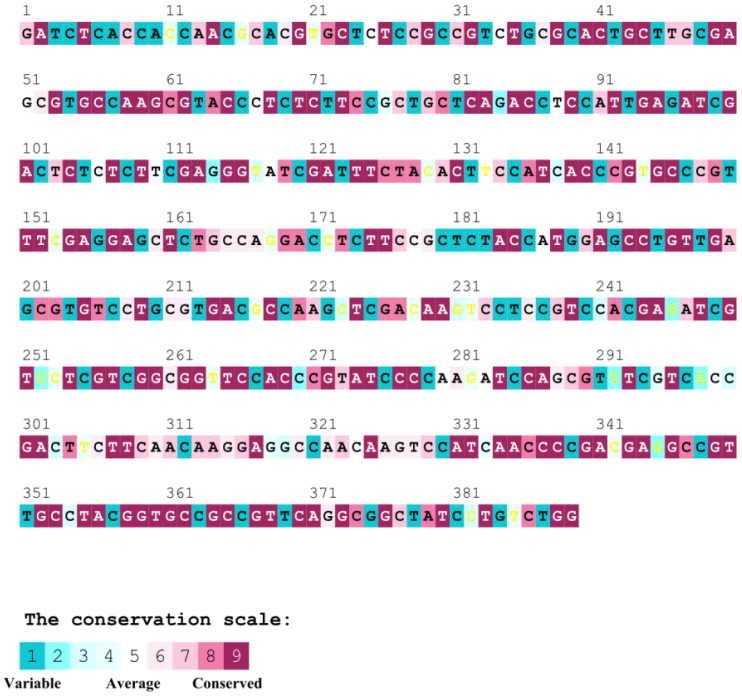

Fig. 2.Conservation scores of each nucleotides of native *A. fumigatus* HSP70 sequence by ConSurf online tool. Each color was characterized as a score. Scores were calculated based on pair wise alignment of all sequences (99 sequences) with native *A. fumigatus* HSP70 sequence as query sequence. Calculation for yellow-colored nucleotides was performed on less than 10% of the sequences meaning that the present nucleotides in the column were less than 10% of the total present sites in the column.

Performing similarity analysis on 99 fungal HSP70 sequences using YASS program resulted in 18,405 pair wise alignments used by the program to produce a dot-plot representation. The resulted dot-plot diagram depicts compacted collection of sequences in few areas of the plot indicating low insertion, deletion, repeated regions and nucleotide divergence among aligned sequences (Fig. 3). Also, high conservation of fungal HSP70 aligned sequences was demonstrated in the YASS-produced dot-plot by high accumulation of the calculated dots in few limited regions of the plot (Fig. 3).

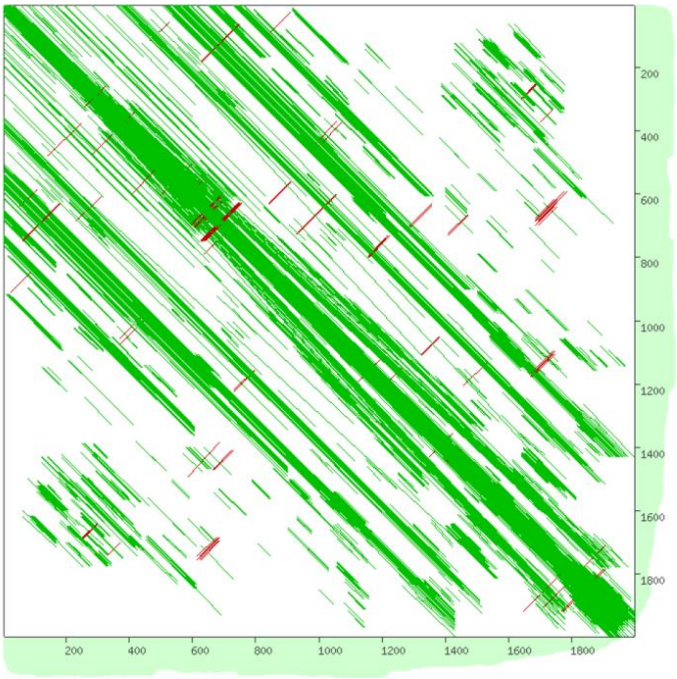

Fig. 3. Dot-plot presentation of HSP70 sequences of important fungal species was produced by YASS sequence similarity search tool. The plot was produced by local pairwise alignment of each two sequences. During the alignment process, each pair of nucleotides from two sequences produced a dot in the plot. X and Y axes represent sequence length and nucleotide sites. Red dots represent reverse alignments and green dots represent forward alignments.

Phylogeny and total mean distances

Phylogenetic tree analysis showed that different *Aspergillus* spp. and *A.fumigatus* strains were placed in the same group of branches with high grades of similarity. Also, among the *Aspergillus* genera, the most similar branches to our sequence belonged to *A. clavatus*, *Aspergillus oryzae*, *A. flavus*, *A. niger*, *A.nidulans* and *A.terreus*, respectively (Figure 4). Furthermore, based on the analyzed sequences, the most similar genus to *Aspergillus* is *Penicillium* spp. Moreover, *Candida* spp. were more distant, because of their less similarity (Table 1) to other fungi involved in this tree (Fig. 4). Clustered arrangements of branches on tree and low distance measures represent the high conservation of the HSP70 gene in the fungi. Total mean distances of the assessed sequences of *Aspergillus* genus and other species were calculated (Table 1). The results of analysis of sequences showed that the distances between sequences of *Aspergillus* and other fungal genera are less than the distances within the *Aspergillus* genus.

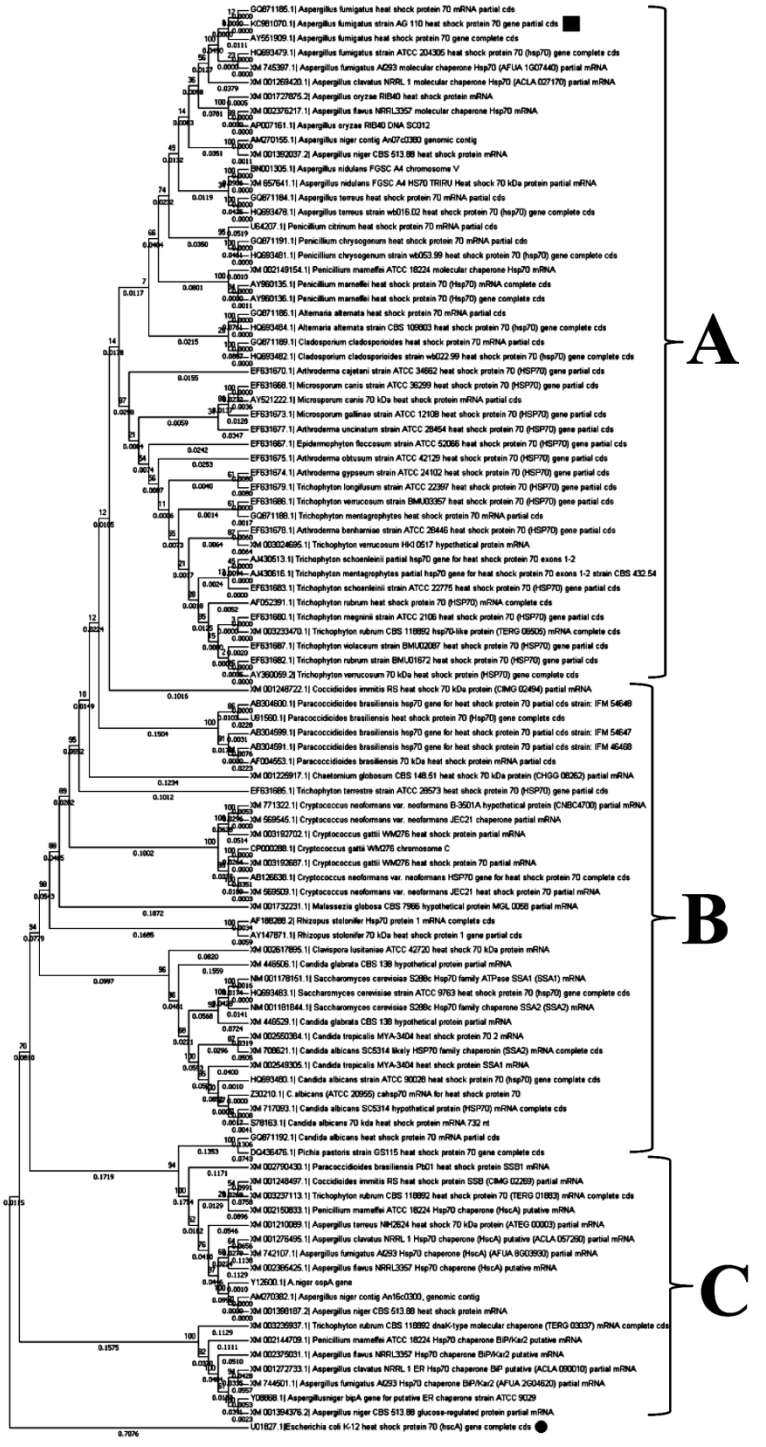

Fig. 4. Phylogenetic tree of HSP70 gene was constructed using sequences of HSP70 family of genes in different fungal species. Our native *A. fumigatus* sequence is marked by ■ and out-group (*Escherichia coli*) is marked by ● on the tree. Units at the bottom of the tree indicate number of substitution events. Length of each pair of branches represents distance between sequence pairs. Sequence information at the tips of branches includes an accession number and strain name for each sequence. Group A of tree branches indicates SSA and HSP70 sequences belonging to *Aspergillus*, *Penicillium*, *Alternaria, Cladosporium, Microsporum*, *Trichophyton* or *Epidermophyton* genera. Group B represents SSA and HSP70 sequences which most of them have been taxonomically ordered in yeasts, yeast-like and dimorphic endemic yeasts genera, such as *Coccidioides*, *Paracoccidioides*, *Cryptococcus* and *Candida.* And group C exhibits SSB, HscA, bipA and Kar2 sub-families of HSP70 family of genes belonging to different fungal genera and specie, for instance *Aspergillus*, *Coccidioides* and *Penicillium marneffei.*

Table 1. Average evolutionary divergence of sequence pairs is calculated by MEGA 6.0.6 in *Aspergillus* genus and all other fungal sequences.

Gene name	Organisms	Total mean distances[a]	Standard error estimates (S. E.)	Selected Variance estimation method	Selected Substitution mode for Amino acid
HSP70	Other fungi (those that are presented in the tree)	d = 0.198	0.017	Bootstrap method	Poisson model
HSP70	Aspergillus genus	d = 0.076	0.0011	Bootstrap method	Poisson model

[a] Total mean distances of sequences in *Aspergillus* genus and other species.

Discussion

HSP70 has been known as an important agent in pathogenicity and immunogenicity in fungal cells. The responses of fungi could be broadly divergent and unpredictable, because regulation of stress-related genes or pathways is strictly related to environmental stimuli, like chemical, nutritional and hormone signals (Fassler and West, 2011). *A.fumigatus* could tolerate high temperature up to 60°C and it is more known to be a ubiquitous environmental fungus (Beffa et al., 1998; Latge, 1999; Holder and Lewis, 2003) unlike the *Candida* spp. which is known to be a commensal normal flora (Spicer, 2008). The importance of HSP70 family members in folding, transporting and maintaining of cell proteins while in tense conditions, was emphasized by prior studies (Zugel and Kaufmann, 1999; Nollen and Morimoto, 2002; Mayer and Bukau, 2005). These attributes could lead to the survival of pathogen within the host body as a result of keeping proper function of proteins involved in pathogenicity and homeostasis of microorganism. HSP70 sequence exhibits high levels of conservation along the *Aspergillus* genome. High degree of sequence conservation in HSP70 might be an advantage which makes it suitable for phylogenic studies (Zhang et al., 1998).

Researchers agree that a phylogenetic tree showing the similarities, relationships and distances, could be important part of research in many areas of biology, such as systems biology and evolutionary biology. Comparing genes or protein sequences in a phylogenetic context can provide deep insight into their function and evolutionary patterns (St-Germain and Summerbell, 2003). DNA sequencing and phylogenetic analysis could accurately be employed to identify and classify fungi. Some obscurities in this field have already been studied to efficiently identify and classify the fungi. For instance, the amino acid sequences of Alkaline/Serine protease and 28s rDNA from allergenic fungi have been surveyed (Burnie et al., 2006). Phylogenetic relationships based on 28s rDNA among different fungi showed that *Penicillium* spp. and *Aspergillus* spp. were placed closely in the tree, although other *Eurotiomycetes* were in near branches with them. However, *Candida* spp. were located in a separate branch, (Burnie et al., 2006).

In Iran, *A. fumigatus* is known as predominant mold in the environment, but there are not enough data about its virulence genes. The sequence of these genes has not yet been completely determined and further studies are needed in this field. In this study, the presence of HSP70 gene in a native *A. fumigatus* was confirmed and its partial sequence was identified. We had limitations in retrieving large collection of *A. fumigatus*

HSP70 gene sequences from GenBank databases, because there were only five sequences of *A. fumigatus* HSP70 gene in the GenBank and the identity ratio of all the five sequences when compared with each other in the BLAST analysis was 100%. In considering the fact that these five sequences were from four different geographical regions (USA, Austria, India and Iran) and submitted by four different research groups, high identity ratios from BLAST analysis could not be as result of a bias or wrong submission of data or inaccurate sequencing procedure. Instead, it could indicate very high intra-species conservation of HSP70 gene as further approved by our phylogenetic, similarity and conservation analyses.

In this mean, the conservation ratio and similarity of fungal HSP70 sequences were assessed using online analysis tools to provide complementary and supporting data to our phylogeny analysis. Conservation and similarity analysis showed high conservation ratio and similarity in fungal HSP70 gene sequence. Moreover, phylogenetic relationships between native *A. fumigatus* and the other fungi revealed that the HSP70 sequence variation was low within the *Aspergillus*, *Penicillium* and *Trichophyton* species, but the variation inter-species was high enough to approve their separation in tree branches localization. The results of inspections about the HSP70 variability suggest high intra-species similarities but divergence inter-species among fungi belonging to *Aspergillus* and *Penicillium* genera. *Aspergillus* is an anamorphic genus, consisting of nearly 837 species classified into almost ten disparate teleomorphic genera (Latgé, 1999; O'Gorman, 2011). In our study, the most similar *Aspergillus* spp. to the native *A. fumigatus* were *A. clavatus* and *A. niger* (with 94% identity) followed by *A. terreus* (with 93% identity). But *A. nidulans* had less similarity (88% identity). The branch pattern of *Aspergillus* spp. on the constructed phylogenetic tree is also indicating the above mentioned identity ratios These findings are compatible to the results of other researchers indicating that *A. nidulans* is a member of the teleomorphic genus *Emericella*, but *A. fumigatus* belongs to the genera *Neosartory* (Goodley et al., 1994).

But on the resulted phylogenetic tree, some of the fungal species were placed on different branches of clades. For example, *A. flavus*, *A. clavatus*, *A. niger* and *A. fumigatus* were placed on different clades on the tree. This is also true for some other fungal species, such as *Trichophyton rubrum* and *P. marneffei.* The explanation to this result is that the sequences were collected and integrated into our phylogeny analysis from all members of HSP70 family of proteins (SSA, SSB, SSC, SSD) to also assess their sequence relationship. Generally, HSP70 family of proteins is known to be highly homologous (Daugaard et al., 2007). In *E. coli*, the major HSP70 proteins are dnaK and later discovered hscA and hscC. DnaK, hscA and hscC are HSP70 homologs in *E.coli* (Lelivelt and Kawula, 1995; Hesterkamp and Bukau, 1998; Itoh et al., 1999). There are also at least ten members of HSP70 proteins in yeasts encoded by five groups of genes consisting of SSA, SSB, SSC, SSD (kar2) and SSE. SSA and SSB proteins are cytosolic and their sequence is approximately 60% identical (Craig et al., 1995; Sharma and Masison, 2009). Kar2 gene of yeast is the homolog of mammalian BiP and both names have been used to indicate same gene (Normington et al., 1989; Jung et al., 2013). Considering the mentioned data grouping of SSA or Kar2 sequences in the same clades of phylogenetic tree while separated from other sequence

clades could be explained to be as a result of their sequence differences. Also, *E. coli* branch separated from all the fungal branches indicates that its sequence differences were high enough to be differentiated from the protein subclasses in fungal genera.

E. coli as a prokaryote demonstrated a complete out-group attribute implying the parallel evolution to fungi as eukaryote. However, sequence alignment supports the proposal that there is extensive structural conservation in the HSPs from prokaryotes to eukaryotes (St-Germain and Summerbell, 2003).

The impact of environmental factors, stress stimuli, or living location of microorganisms in triggering evolutionary changes could be subjected to survey in phylogeny and evolutionary studies. Therefore, more precise and deep studies should be done to clear the obscurities of how evolution occurs in fungi, what is the relation between environmental changes and evolution and whether the evolution could incorporate in virulence and pathogenicity. Also, more studies and data are required to employ phylogenetic analysis of HSP70 as a tool to investigate molecular evolution among eukaryotic cells. The most important conclusion from HSP70 sequence analysis involves the monophyly of the fungi, which is supported by high bootstrap values in neighbor-joining analysis and specific phylogenetic relationship among fungi. These results support the present taxonomic classification of fungi and close relationship between native *A. fumigatus* and other strains of species and fungal genera worldwide (Dagenais and Keller, 2009). As regards to previous studies, single-gene based analysis, would not solve all ambiguities or represent evolutionary history of species. It is expected that recent knowledge acquired by innovative genomic researches in fungi could be advantageous in future studies. Such valuable data may be able to provide the remarkable progress in future phylogenetic studies.

Briefly, as expected, *Penicillium* spp. were the most phylogenetically relevant species to *Aspergillus* spp. Other molds were also placed in close clades on the constructed phylogenetic tree. Yeasts and yeast-like fungi were placed in more distanced clades. *E. coli* as a prokaryote was totally placed in a separate branch from all the fungi. These differences may assist researchers to find more specific targets of novel antimicrobials and also depict a deeper map of fungal genome differences. In addition, more comprehensive data about fungal HSP70 gene sequence differences may assist to better understand thermo-resistance in different fungal species.

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