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Posted Date: 30 September 2024

doi: 10.20944/preprints202409.2323.v1

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Phylogenetic *In Silico* Analysis Indicates that the Moonlighting 14-3-3 Protein of *Ajellomycetaceae* Family and *Homo sapiens* Share Conserved and Distinct Features in Their Peptide Binding Site

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Abstract: The 14-3-3 protein in dimorphic fungi from the *Ajellomycetaceae* family, including *Blastomyces* spp., *Histoplasma* spp., *Paracoccidioides* spp., *Emergomyces* spp., and *Emmonsia* spp., play a crucial role in host-pathogen interactions. Thus, we performed a comparative genomic analysis across 21 fungal species which revealed the presence of two 14-3-3 gene copies in most fungi, with exceptions in *Histoplasma ohiense* and *Emergomyces africanus* which revealed one and three gene copies, respectively. Moreover, in this study it was found a high degree of similarity (over 70%) between fungal 14-3-3 proteins and the human 14-3-3ε isoform, particularly in conserved Ser/Thr phosphorylation sites. However, distinct differences were noted in the C- and N-terminal regions, where fungal 14-3-3 proteins have additional amino acids. Though not directly linked to pathogenicity, these structural differences may offer insights into new therapeutic strategies. The conserved regions and 3D similarities identified could serve as potential targets for broad-spectrum antifungal drug development and vaccine production. Further research is necessary to validate these *in silico* findings and explore the functional roles of these phosphorylation sites, which could contribute to understanding fungal pathogenicity and developing specific antifungal interventions.

Keywords: 14-3-3 protein; dimorphic fungi; *Ajellomycetaceae* family; phylogenetic analysis; OrthoFinder; Swiss-model

1. Introduction

The eukaryotic protein 14-3-3 assumes several multifaceted cellular tasks, manifesting its characteristic moonlighting behavior. Within fungal kingdom, the functional repertoire of 14-3-3 encompasses various pivotal roles; these include involvement in several processes as follow: virulence modulation (Rodríguez-Romero et al. 2019; Marcos et al. 2016), adhesion mechanisms (Assato et al. 2015; Marcos et al. 2016), orchestration of growth dynamics (Sun et al. 2018), regulation of cell signaling pathways and cycles (Lottersberger et al. 2006), modulation of carbon metabolism (Y. Wang et al. 2004), facilitation of filamentation and ascospore formation (Herod et al. 2022), DNA duplication (Kumar 2018), cellular survival mechanisms under stressful conditions (Sun et al. 2018),

 doi:10.20944/preprints202409.2323.v1

cell secretion (Gelperin et al. 1995), coordination of cell wall composition and chitin synthesis (Lottersberger et al. 2006), aggresomal assembly (Xu et al. 2013), regulation of the spindle assembly checkpoint during cell division (Caydasi et al. 2014), modulation of retrograde signaling pathways (Trendeleva and Zvyagilskaya 2018), and participation in the intricate regulation of apoptosis (Tohru Ichimura et al. 2004).

The 14-3-3 protein exhibits diverse isoform variability across various fungi genera and species and can be expressed through various gene translations. Notably, this protein can range from a singular isoform in fungi like *Paracoccidioides brasiliensis* (Assato et al. 2015) and *Candida albicans* (Cognetti, Davis, and Sturtevant 2002; Palmer et al. 2004), seven isoforms in *Homo sapiens* (T Ichimura et al. 1988), to a more extensive repertoire, as observed in the plant species *Arabidopsis thaliana*, which boasts a total of 13 distinct isoforms (Wilson, Swatek, and Thelen 2016), highlighting the considerable diversity and complexity present within the 14-3-3 protein family across different organisms.

The Onygenales order within the *Ajellomycetaceae* family encompasses several pathogenic dimorphic fungi such as *Histoplasma*, *Paracoccidioides*, *Emergomyces*, *Emmonsiellopsis*, *Emmonsia*, and *Blastomyces*, all of which share similar characteristics concerning host-pathogen interactions and possess a distinctive ability to undergo morphological shifts in response to environmental cues during their transition from the environment to the host system (Gauthier 2015). This classification underscores commonalities among these fungi in terms of their behavior and interactions with host organisms (Muñoz et al. 2018), in which 14-3-3 protein plays a pivotal role.

Recently, it has become evident that climate change due to human activity is creating conditions conducive to the emergence of new human fungal pathogens. These changes increase virulence, fungal dispersal, vectors, geographic range, and host susceptibility (Nnadi and Carter 2021). These scenarios, coupled with the growing resistance of fungi to available antifungal drugs (Fisher et al. 2022), underscore the urgent need for new specific targets.

In this context, phylogenetic analysis is a crucial bioinformatic tool enabling a comprehensive view of organisms' evolutionary trajectories over time *in silico*. This analytical approach unveils genetic relationships and similarities among different species, shedding light on their evolutionary history and providing valuable insights into their genetic makeup (Burr 2010). It is possible to evaluate similarities and differences among species that can be applied as diagnostic markers, sequences used in broad-spectrum fungal identification analysis, and the development of new broad-spectrum drugs and vaccines (Simonson et al. 2021; Van Burik et al. 1998; Taborda and Camargo 1994).

Thus, in this study we aimed to compare 14-3-3 orthologs within the genome of the *Ajellomycetaceae* family and *Homo sapiens*' isoforms seeking to yield novel insights that could be show in the protein functions of a new shared and specific targeting for broad-spectrum fungal sequences, drug-specific targets, and vaccine development to infections caused by dimorphic fungi.

2. Results

We conducted a comparative analysis of 21 species, examining a total of 193,630 genes. Our investigation revealed the presence of 181,719 orthogroup genes, constituting 93.8% of the total, distributed across 13,770 orthogroups. Additionally, we identified 11,911 unassigned genes, comprising 6.2% of the dataset. Within this dataset, 369 orthogroups were specific to particular species, encompassing 1,554 species-specific genes. The average size of orthogroups was 13.2, with a median size of 15.0. Furthermore, our analysis identified 3,839 orthogroups shared among all species, and 2,383 single-copy orthogroups.

In the genomes of the *Ajellomycetaceae* family, we identified two copies of the 14-3-3 gene family, except for *Histoplasma ohiensis* G217B, which possesses only one gene copy, and *Emergomyces africanus* CBS 136260, which harbors a third gene copy (Table 1). The human genome exhibits 17 copies of the gene family (Table 2).

Table 1. Predicted 14-3-3 protein of the *Ajellomycetaceae* family.

Organism name	Accesion Number	Ortholog 1	Ortholog 2	Ortholog 3

 doi:10.20944/preprints202409.2323.v1

Paracoccidioides americana Pb03	GCA_000150475.2_Paracocci_br_Pb03_V2_protein	EEH18728.1	EEH19135.2	
Histoplasma duboisii H88	GCA_000151005.2_ASM15100v2_protein	EGC42823.1	EGC45970.1	
Histoplasma capsulatum H143	GCA_000151035.1_ASM15103v1_protein	EER42730.1	EER43039.1	
Blastomyces silverae UAMH 39	GCA_001014755.1_ASM101475v1_protein	KLJ08234.1	KLJ08684.1	
Emergomyces africanus CBS 136260	GCA_001660665.1_Emmo_afri_EA111_protein	OAX82061.1	OAX82668.1	OAX82671.1
Paracoccidioides venezuelensis Pb300	GCA_001713645.1_ASM171364v1_protein	ODH28354.1	ODH33981.1	
Paracoccidioides restrepiensis PbCNH	GCA_001713695.1_Paracocci_br_PbCNH_protein	ODH47297.1	ODH50940.1	
Emergomyces pasteurianus Ep9510	GCA_001883825.1_Emmo_past_UAMH9510_V1_protein	OJD14033.1	OJD14395.1	
Emmonsia crescens UAMH4076	GCA_002572855.1_Emmon_cres_4076_V1_protein	PGH30391.1	PGH33847.1	
Blastomyces parvus UAMH130	GCA_002572885.1_Emmon_parv_130_protein	PGH00750.1	PGH07673.1	
Helicocarpus griseus UAMH5409	GCA_002573585.1_Heli_gris_5409_V1_protein	PGH01130.1	PGH02170.1	
Polytolypa hystricis UAMH7299	GCA_002573605.1_Poly_hyst_7299_V1_protein	PGH05826.1	PGH15883.1	
Histoplasma capsulatum WU24	GCA_017310585.1_ASM1731058v1_protein	QSS61858.1	QSS62247.1	
Histoplasma ohiense G217B	GCA_017607445.1_ASM1760744v1_protein	KAG5289754.1		
Blastomyces dermatitidis ER-3	GCF_000003525.1_BD_ER3_V1_protein	XP_045274151.1	XP_045275734.1	
Blastomyces gilchristii SLH14081	GCF_000003855.2_BD_SLH14081_V1_protein	XP_002624823.1	XP_002627124.1	
Histoplasma mississippiense NAm1	GCF_000149585.1_ASM14958v1_protein	XP_001540333.1	XP_001540687.1	
Histoplasma capsulatum G186AR	GCF_000150115.1_ASM15011v1_protein	XP_045291077.1	XP_045291482.1	
Paracoccidioides lutzii Pb01	GCF_000150705.2_Paracocci_br_Pb01_V2_protein	XP_002791205.1	XP_002796914.1	
Paracoccidioides brasiliensis Pb18	GCF_000150735.1_Paracocci_br_Pb18_V2_protein	XP_010759301.1	XP_010759842.1	
Paracoccidioides restrepiensis Pb60855	Unpublished	Pb60855_p_5916	Pb60855_p_7352	

Table 2. Predicted 14-3-3 protein of the *Homo sapiens* (Accession number GCF_000001405.40_GRCh38.p14_protein).

Gen copy	Accession Number
Ortholog 1	NP_001129171.1
Ortholog 2	NP_001129172.1
Ortholog 3	NP_001129173.1
Ortholog 4	NP_001129174.1
Ortholog 5	NP_003395.1
Ortholog 6	NP_003396.1
Ortholog 7	NP_003397.1
Ortholog 8	NP_006133.1
Ortholog 9	NP_006752.1

 doi:10.20944/preprints202409.2323.v1

Ortholog 10	NP_006817.1
Ortholog 11	NP_036611.2
Ortholog 12	NP_647539.1
Ortholog 13	NP_663723.1
Ortholog 14	XP_005251118.1
Ortholog 15	XP_005251120.1
Ortholog 16	XP_016869299.1
Ortholog 17	XP_016869300.1

The first 14-3-3 gene copy within the *Ajellomycetaceae* family exhibited a closer phylogenetic relationship to the human 14-3-3 protein ε (Figure 1) (Ortholog 9, Table 2). Upon alignment of 14-3-3 orthologs, the sequence displayed a conserved peptide binding site from the human 14-3-3 protein ε across all fungal proteins. However, polymorphic sites were observed in the dimer interface of the second gene copy (Figure 2).

Regarding phylogenetic analysis, the first 14-3-3 gene copy (Figure 1, blue) formed a clade comprising orthologs from the genera *Histoplasma*, *Blastomyces*, *Emergomyces*, and *Emmonsia*, with the *Paracoccidioides* genus as the external group. Conversely, the second gene copy (Figure 1, red) formed a clade with the *Paracoccidioides* genus and other genera, while the *Histoplasma* genus served as the external group. Furthermore, the second copy of the 14-3-3 gene found in the *Ajellomycetaceae* family also demonstrated a common ancestor with *Homo sapiens* 14-3-3 ε isoform.

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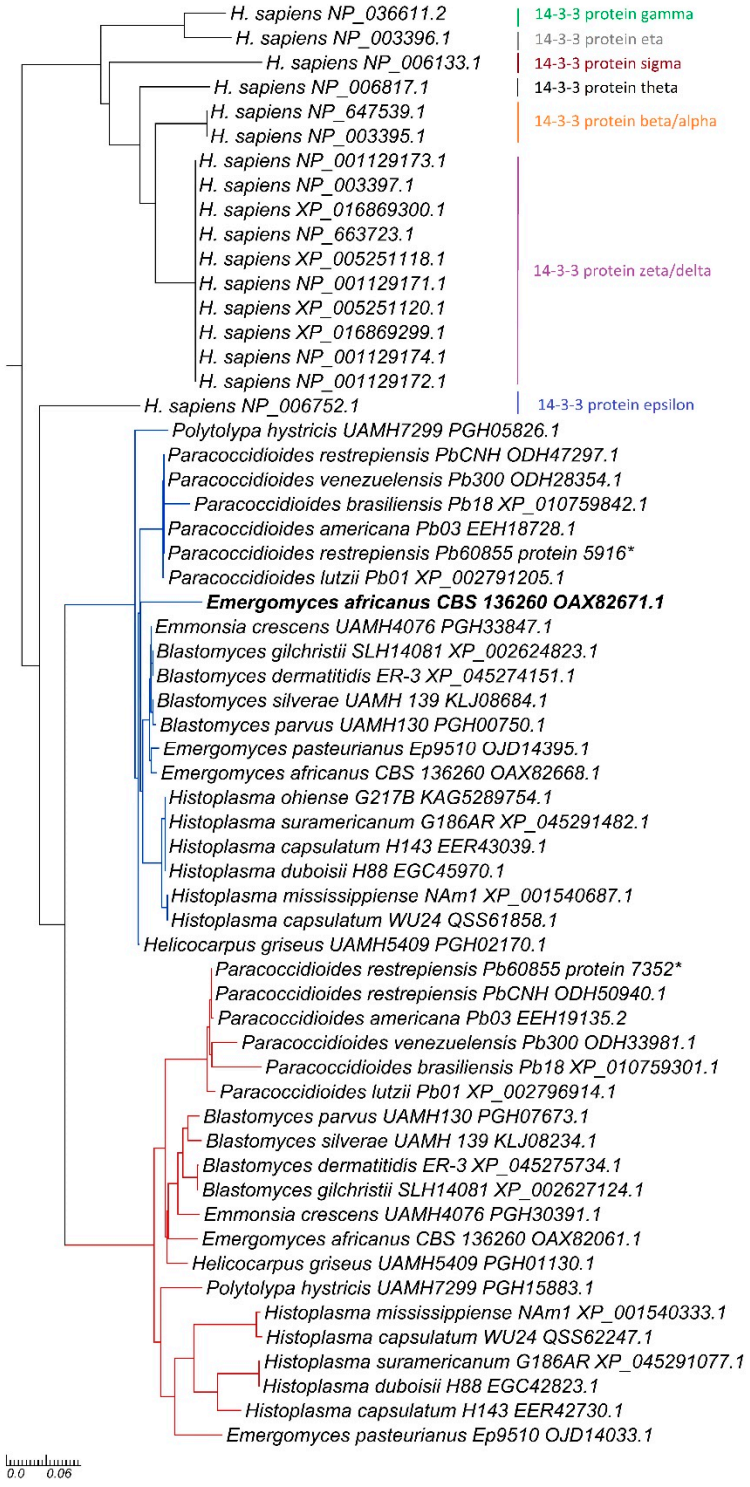

Figure 1. Gene tree of 14-3-3 ortho-group inferred by OrthoFinder. The 14-3-3 tree from the *Ajellomycetaceae* family (red and blue lines) was rooted within the human 14-3-3 family. In these fungi, a duplication event is observed, which generates two gene copies. The first gene copy (blue line) is present in all fungi of this family. *Emergomyces africanus* CBS 136260 exhibited a third copy in this orthogroup (bold). In the second gene copy group (red line), *Histoplasma ohiense* G217B is not present. The protein accession number is shown for each sequence. *Sequences from the lab isolated are not available yet.

When the resulting 14-3-3 alignment was visualized in Geneious Prime V 2023.0 (Figure 2), conserved and distinct amino acids were identified in the *Ajellomycetaceae* fungi and *Homo sapiens* sequences. Additionally, the fungal copies were distinct from each other and differed when

 doi:10.20944/preprints202409.2323.v1

compared to the *Homo sapiens* copy. Nevertheless, an extensive conserved sequence was also visible among all sequences. The mean length of the 14-3-3 protein was 268 amino acids, with 29.9% (104) identical sites among all species analyzed and a pairwise identity of 71.9%. Furthermore, amino acid groups comprised 18.2% acidic, 13.4% basic, 31.6% charged, 25.5% polar uncharged, 43.7% hydrophobic, 24.4% GC-rich, and 21.5% AT-rich amino acids. Finally, the frequency of Serine (Ser) was 7.7% (1,178 frequency), and Threonine (Thr) was 4.7% (712 frequency). The mean molecular weight was 29.936 kDa, the mean isoelectric point was 4.47, and the mean absorbance of 280 nm of 1mg/mL was 0.93 AU.

Figure 2. Sequence alignment of human 14-3-3 proteins with *Ajellomycetaceae* family. Sequence alignments were performed using the Clustal Omega program and visualized in Geneious. The dimer interface sites [polypeptide binding] from human 14-3-3ε protein are highlighted.

 doi:10.20944/preprints202409.2323.v1

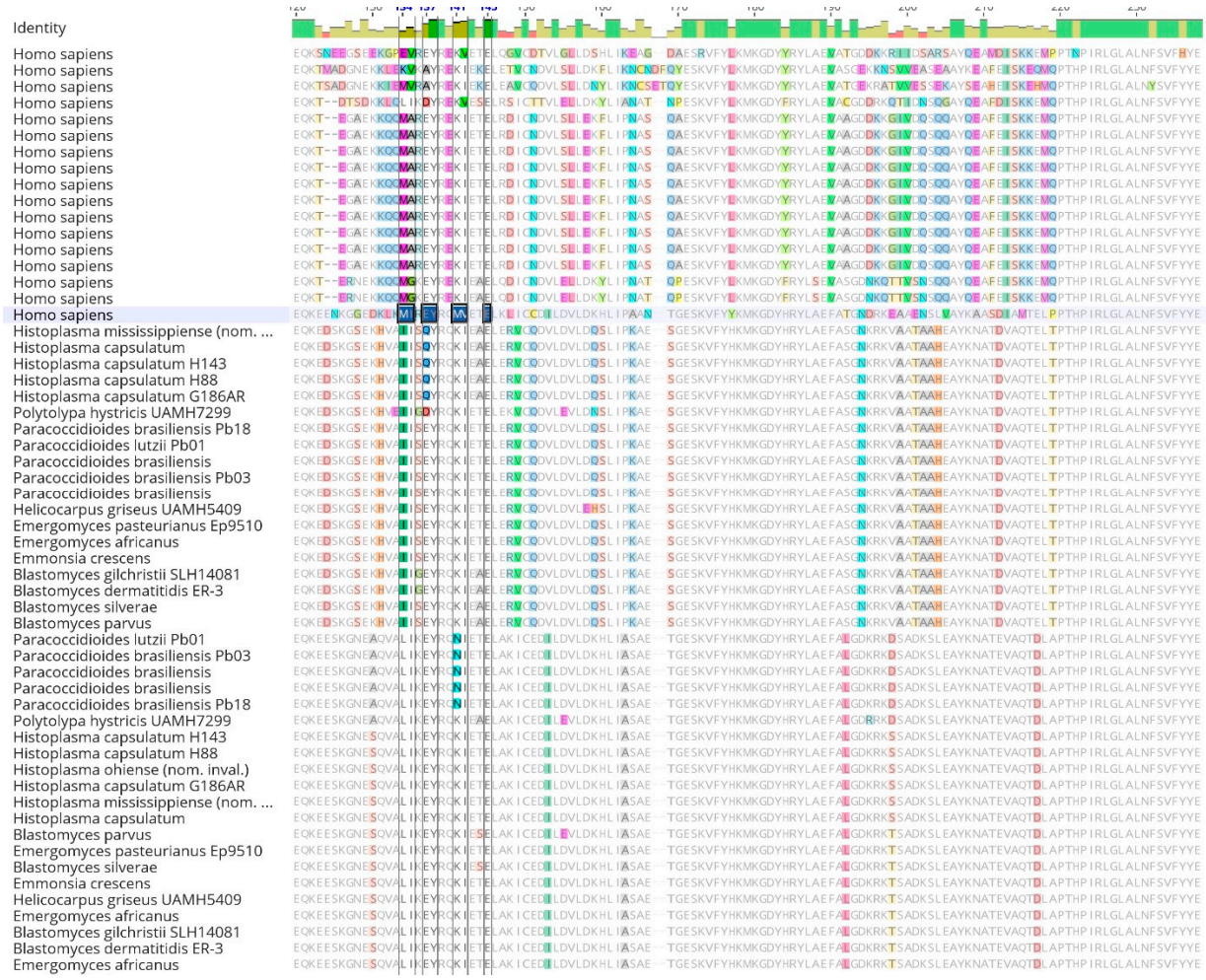

Figure 2. (continuation). Sequence alignment of human 14-3-3 proteins with *Ajellomycetaceae* family. Sequence alignments were performed using the Clustal Omega program and visualized in Geneious. The dimer interface sites [polypeptide binding] from human 14-3-3ε protein are highlighted.

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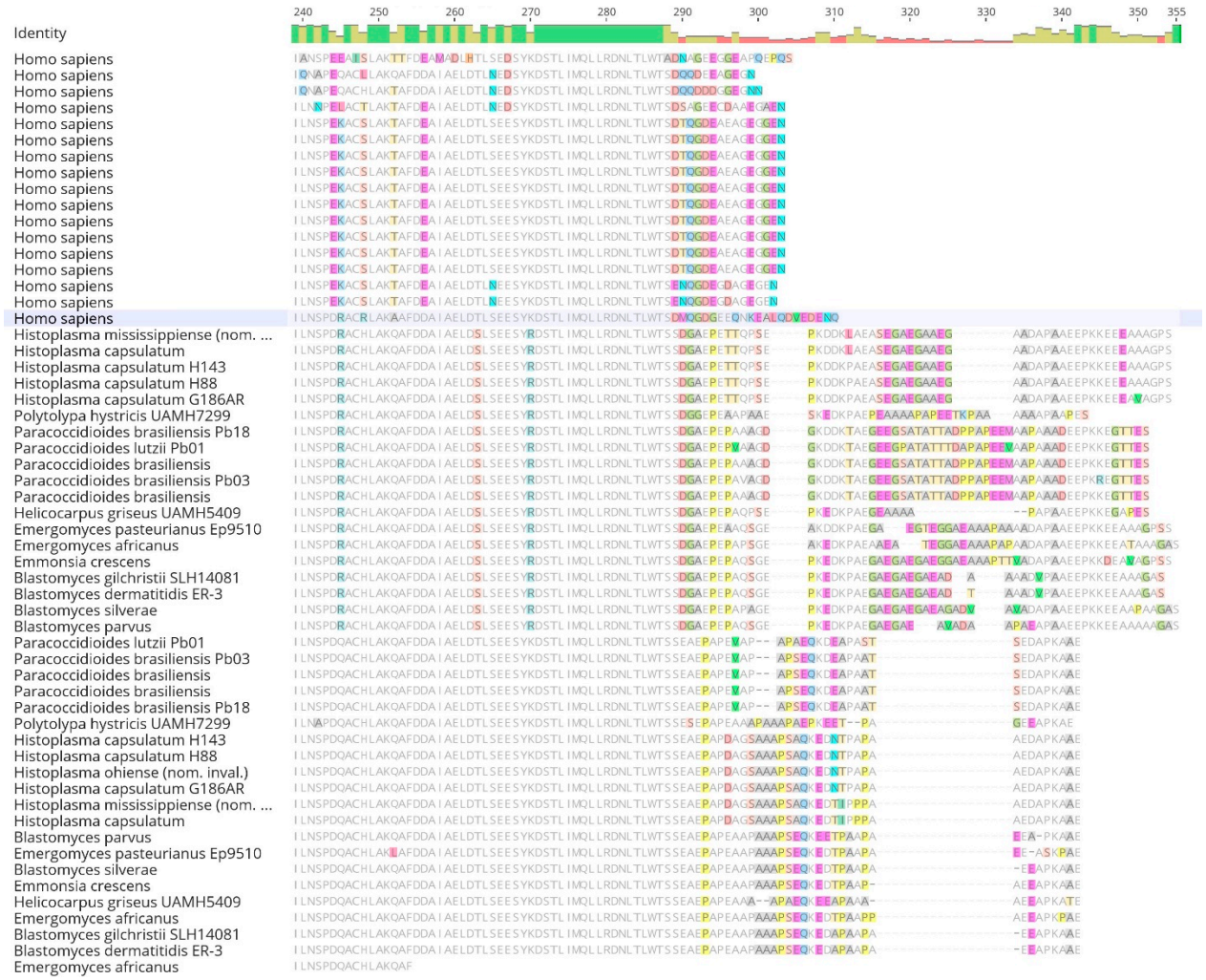

Figure 2. (continuation). Sequence alignment of human 14-3-3 proteins with *Ajellomycetaceae* family. Sequence alignments were performed using the Clustal Omega program and visualized in Geneious. The dimer interface sites [polypeptide binding] from human 14-3-3ε protein are highlighted..

Protein modeling was created with Swiss-model considering oligo state as a monomer, GMQE (Global Model Quality Estimate) ≥ 0.80, 0.85 < coverage < 1.0, QMEAN Z-score < 1, and 86.72.0 < sequence identity < 100. Figure 3 represents protein 3D models for each *Ajellomycetaceae* family fungus.

 doi:10.20944/preprints202409.2323.v1

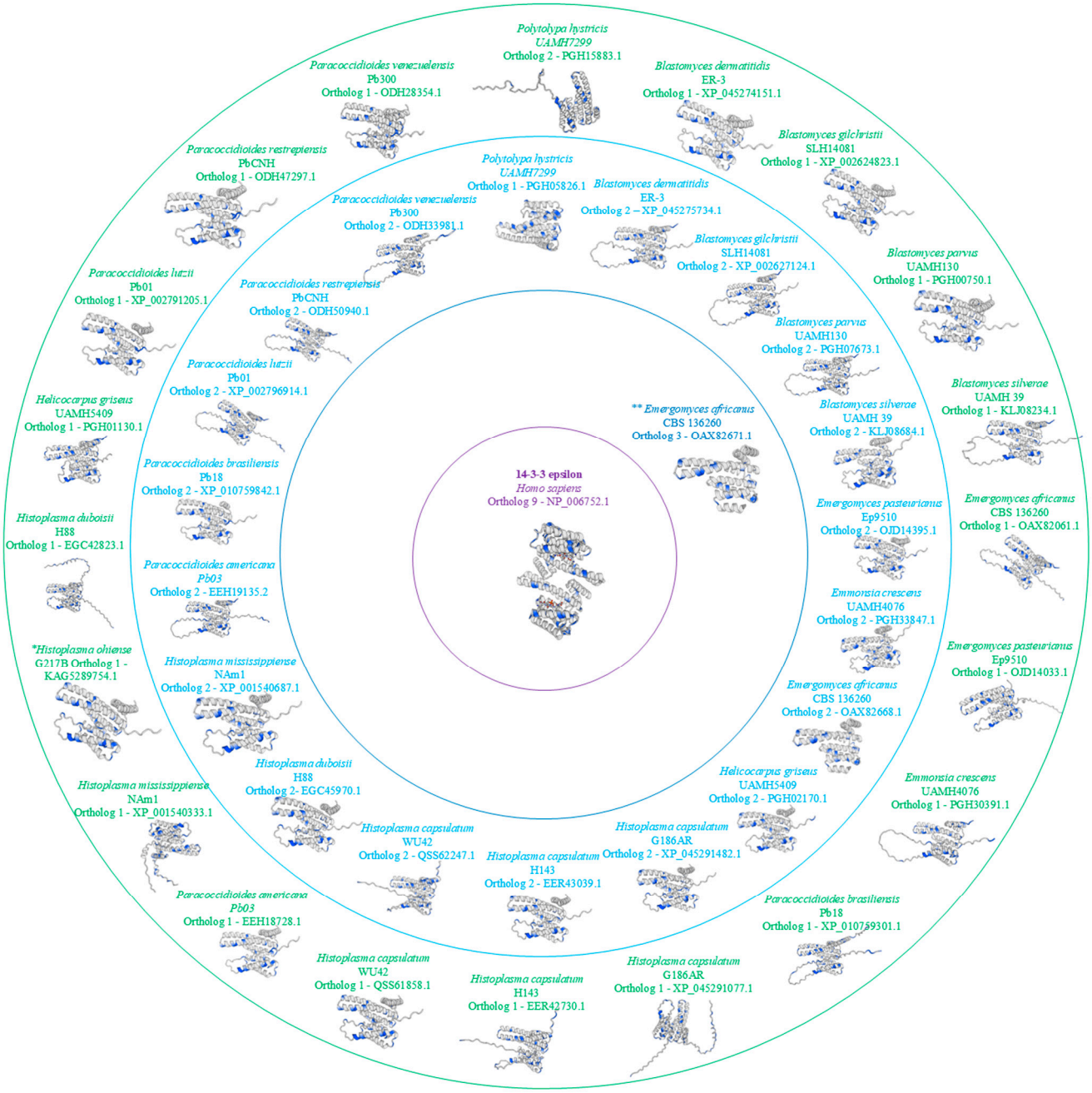

Figure 3. 3D Model of *Homo sapiens* **14-3-3ε Protein and Orthologs from the** *Ajellomycetaceae* **Family.** Serine/threonine (Ser/Thr) residues are highlighted in blue in the 3D model, with ortholog 1 shown in green, ortholog 2 in light blue, ortholog 3 in dark blue, and the Homo sapiens 14-3-3ε protein depicted in purple. * Only one ortholog. ** Three orthologs.

Through the 3D analysis of the 14-3-3 protein in fungi from the *Ajellomycetaceae* family, a high degree of similarity with the human 14-3-3Ɛ protein is observed, with similarity exceeding 70%, depending on the species analyzed. However, one noticeable difference is the more significant number of amino acids at the C- and N-terminal ends of the fungal 14-3-3 protein. This difference has not been demonstrated to have any relation to the pathogenicity of these fungi.

3. Discussion

 doi:10.20944/preprints202409.2323.v1

The 14-3-3 protein is categorized as a prominent class of molecular chaperones, interacting with over 200 protein targets and engaging a diverse array of binding partners involved in cellular signaling pathways (Hondermarck 2010), exhibiting closer evolutionary proximity among human protein to yeasts and plants than to other animals, suggesting a shared ancestral protein function (Wang and Shakes 1996). The multiple binding partners of 14-3-3 are due to the phosphorylation majority, therefore the interaction with phosphatases and kinases modulates its phosphorylation, which has been related to various cell pathways of differentiation, growth, migration, and survival (Hondermarck 2010).

All isoforms of 14-3-3 proteins contain conserved serine/threonine (Ser/Thr) sequence motifs, with phosphorylation necessary to activate and bind f the protein C-termini locus. While certain assumptions can be inferred from the presence of 14-3-3 motifs, the sequences of binding proteins alone cannot serve as a sole predictive factor (Liu et al. 2021; Fu, Subramanian, and Masters 2000).

In human cells, among 14-3-3 isoforms, the 14-3-3ε, known as tyrosine 3/ tryptophan 5-monooxygenase activation protein epsilon due to its activity, is mainly expressed in lymphoblasts, brain, heart, testes, and adipocyte cells, been also related to a negative androgen regulator mostly found in mitochondria during steroidogenesis, although this isoform can be found in almost all tissues (Muslin et al. 1996; Aitken et al. 2002; Qu et al. 2022; Zhang et al. 2024).

The specificity of 14-3-3ε is intricately linked to various physiological mechanisms, including cellular proliferation, signal transduction, cell cycle regulation, apoptosis, autophagy, intracellular electrolyte balance, cardiac morphogenesis and repolarization, neurodevelopment, and innate immune responses. As such, 14-3-3ε assumes significance in the progression of cardiovascular diseases, neurodegenerative disorders, cancer, and inflammatory conditions (Zhang et al. 2024). Although the 14-3-3ε is a cytoplasm protein, it can also be present in the nucleus, Golgi apparatus, mitochondria, and plasm membrane (Celis et al. 1990; Guo et al. 2014).

Noteworthy, the human 14-3-3ε regulates cell innate immunity through T cells activation. The SH2 domain-containing leukocyte protein of 76 kD (SLP-76) during viral infections regulates T-cell signaling. The binding of phosphorylated- SLP-76 to 14-3-3ε results in a negative signal to regulate T cells activation (Bartolo et al. 2007; Iyer et al. 2022), leading to a dumped immune response.

Similarly, this protein has a moonlighting characteristic in fungi cells due to its wide range of cell signaling pathways. For fungi, the 14-3-3 closed to human 14-3-3ε protein (Fig. 1) exhibits an important role during host-pathogen interaction, acting as a critical virulence factor. Studies conducted by silencing 55% of 14-3-3 protein (NCBI XP_010759842.1) in *Paracoccidioides* (Pb14-3-3 aRNA) have demonstrated significant enhancement of survival of pneumocytes, implying that the improved survival is a result of reduced interaction between the fungi and the host plasma membrane, increasing *Galleria mellonella* larval survival in 35% when infected with silenced strain (Marcos et al. 2016; 2019). Furthermore, recent studies highlighted its importance during biofilm formation in *P. brasiliensis* (Sardi et al. 2024). According to this study, wild *P. brasiliensis* (Pb18) increases its enolase, 14-3-3, and GAPDH gene expression, while the Pb14-3-3 aRNA strain shows a decrease in the expression of these genes, demonstrating that 14-3-3 could influence the expression of other biofilm-related genes (Sardi et al. 2024).

The reduction in its interaction with host cells appears to be mediated by the modulation of the 14-3-3 protein in the TLR signaling pathway (Bonfim, Mamoni, and Lima Blotta 2009). Specifically, it binds to TLR2, TLR3, TLR4, TLR7/8, and TLR9, iproducing pro-inflammatory cytokines such as IL-6, TNFα, and IFN-β. This binding dampens the pro-inflammatory response, inhibits nitric oxide (NO) production, and suppresses the activation of IFNγ/CD8/T cells and IL-17/CD8/T cells, along with cytotoxic functions, including the downregulation of granzyme B and perforin. Notably, the binding of 14-3-3 to TLR3 has recently been proposed as an evasion mechanism (Jannuzzi et al. 2019; Bonfim, Mamoni, and Lima Blotta 2009).

It is crucial to emphasize that, in the case of other members within the *Ajellomycetaceae* family, existing literature lacks data showcasing the binding of the 14-3-3 protein to TLR receptors. Despite observed differences in peptide sequences (Fig. 2), notable similarity persists among the studied 14-

 doi:10.20944/preprints202409.2323.v1

3-3ε-like protein of dimorphic fungi, indicating maybe a shared target, indicated by conservative Ser/Thr residues among these fungi family (Fig. 3).

Consequently, we hypothesize that moonlight proteins, such as enolase, Hsp60 (de Matos Silva et al. 2024), and 14-3-3, are virulence factors in dimorphic fungi related to evasion mechanisms and biofilm formation (Marcos et al. 2016; Sardi et al. 2015; Silva et al. 2013). The phylogenetic analysis of the second copy of the 14-3-3 protein within the *Ajellomycetaceae* family revealed a shared ancestor with human isoforms (Fig. 1, red), and sequence alignment further highlighted significant distinctions among the sequences identified in the *Ajellomycetaceae* family.

Despite *Paracoccidioides* and *Histoplasma* belonging to different clades, this divergence may lead to variations in the evolution of these microorganisms, influencing pathogenicity, host-pathogen interaction specificity, and genetic diversity within the 14-3-3 protein family. It is known that population and comparative studies among the *Ajellomycetaceae* family have demonstrated gene expansions and contractions resulted in enhanced or decreased virulence (Muñoz et al. 2016; 2015; Desjardins et al. 2011), creating the hypothesis that these events could be related to evolutionary mechanisms of adaptation in dimorphic fungi (Muñoz et al. 2018).

In our analysis, we utilized both pathogenic and rarely pathogenic dimorphic fungi from the *Ajellomycetaceae* family, along with non-dimorphic species such as *P. hysterics* and *H. griseus* were also present. The results demonstrated the presence of two copies in most analyzed species, suggesting that the 14-3-3 protein is essential not only for host-pathogen interactions and as an evasion mechanism, as hypothesized, but also might function as a non-exclusive pathogenic factor during infection.

Similar observations were made in studies comparing pathogenic and non-pathogenic fungi within the *Ajellomycetaceae* family, indicating the conservation of many switch-related proteins in non-pathogenic fungi, suggesting that these proteins are not solely required for survival in mammalian hosts, thereby not specifying pathogenicity, but also renders them necessary for environmental survival during the mycelial phase (Muñoz et al. 2018). A parallel scenario is observed in *Cryptococcus neoformans*, where the alpha mating factor contributes to mouse virulence and amoebae survival (Nielsen et al. 2005).

The divergence in both gene and protein expression of the 14-3-3 second copy may suggest an evolutionary mechanism of differentiation among species within the *Ajellomycetaceae* family. Various mechanisms contribute to genetic differentiation in fungi, including recombination events, gene gain and loss through duplication or excision, gene family expansion and contraction, genome rearrangements, and horizontal gene transfer. Notably, gene duplications tend to occur more frequently in proteins responding to stress rather than those related to metabolism, leading to differential expression rather than the emergence of new protein functions. Consequently, duplication events in proteins associated with multiple pathways ensure comprehensive coverage (Taylor et al. 2017) , conferring survival advantages to fungi possessing multiple gene copies of a moonlighting protein compared to those without one.

Furthermore, the duplication combined of a contracted third copy of the gene observed in *Emergomyces africanus* might result from environmental pressures favoring the survival of this soil fungus to migrate to mammal hosts (Muñoz et al. 2018).

The 3D similarities and conserved Ser/Thr phosphorylation sites demonstrated among *Ajellomycetaceae* 14-3-3 proteins could be employed as an immunogenic protein for the developing new specific drug targets and vaccines. Further studies are necessary to validate our hypothesis and explore these conserved regions' potential in therapeutic applications. Investigating the functional roles of these phosphorylation sites and their interactions with other cellular proteins will be crucial in understanding their contribution to fungal pathogenicity and their viability as targets for antifungal strategies.

4. Methods

To perform the orthologs analysis, we download all predicted proteins of the *Ajellomycetaceae* family (Table 1) and Human (GRCh38.p14) (Table 2) from the representative genomes NCBI

database. All the data used here have been previously published, except the *P. restrepiensis* 60855. We analyzed homology using OrthoFinder version 2.5.4 by default settings (Emms and Kelly 2015). In the ortho-group output, we found the 14-3-3 orthogroup searching the human protein 14-3-3. Then, the resolved gene tree for the protein orthogroup was visualized using Treegrap2. The protein sequences reported in the 14-3-3 orthogroup were aligned using Clustal Omega v1.2.2. Finally, the resultant alignment was visualized in Geneious Prime V 2023.0 (Biomatters, NZ) and 3D protein modeling was obtained with SWISS-MODEL (Waterhouse et al. 2018)

Non-dimorphic species of *Ajellomycetaceae*, such as *Polytolypa hysterics* and *Helicocarpus griseus*, and dimorphic pathogenic fungi including *Blastomyces* spp., *Histoplasma* spp., *Paracoccidioides* spp., *Emergomyces* spp., and *Emmonsia* spp. were used in the analysis. The NCBI protein accession number is referenced in Tables 1 (*Ajellomycetaceae* family) and 2 (*Homo sapiens*).

Authors' Contributions: Samanta de Matos Silva, Oscar Mauricio Guzman, Marco Antonio Rodrigues, Henrique Gomes Martins, and Angel Gonzalez conceived the idea and wrote the first draft of the manuscript; Maria José Soares Mendes-Giannini, Orville Hernandez Ruiz, and Ana Marisa Fusco-Almeida provided critical revision of the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [grant 2022/15826-0 REGULAR, 2022/15233-0 (MAR), 2024/03014-7 (HGM)]; and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) [finance code 001, 88887.600612/2021-00 (SMS), 88887.839588/2023-00 (SMS), 88887.949391/2024-00 (HGM)].

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

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