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Molecular Identification and Phylogenetic Classification of Fungal Pathogens Isolated from Egyptian Arabian Horses

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ABSTRACT

Horse health and welfare can be negatively impacted by skin diseases, which can be made worse by delays in diagnosis and initiation of appropriate treatment plans. Clinical signs of the diseases can often be similar even though the underlying causes can be different, either infectious or non-infectious. Bacteria, parasites, and fungi can all cause infectious skin diseases. Widespread internal infections can also lead to fungal-related skin infections. Accurate identification of the fungal species and a detailed description of the clinical signs are essential for a successful fungal diagnosis. Fungal ribosomal DNA (rDNA) Internal Transcribed Spacer (ITS) regions were employed; these highly variable sequences are crucial for differentiating between fungal species through PCR analysis. In this study, 50 hair samples were collected from Egyptian horses showing clinical signs of dermatophytosis, out of which 28 were positive fungal cultures. While PCR amplification of the ITS regions method showed that 24 amplification-positive samples (16 samples of *Arthroderma sp*. and 8 samples of *Chrysosporium sp*.) were present out of 28 positive cultures. The PCR results indicated amplification in (580 bp) Following a BLASTn alignment with sequences of those closely related species in the same taxa in the GenBank database. All results of the sequences of ITS1-5.8S-ITS4 regions showed similarity to all isolates of amplified sequences. This was confirmed for two types of fungi: *Chrysosporium zonatum* (2 samples) and *Arthroderma multifidum* (6 samples). A bootstrap of 1000 replications for every fungal isolate was also displayed on the phylogenetic tree, along with varying percentage rates among the fungal isolates. This study demonstrated that the obtained isolates' sequences had (100%) similarity with the sequences of numerous species that had previously been identified as *Chrysosporium zonatum* (PP085497 and PP086050) and *Arthroderma multifidum* (PP085493, PP085494, PP085495, PP085496, PP086051, and PP086052). These results indicate that these two fungal species are highly prevalent in Egyptian horse's skin.

Key words: Dermatophytosis, *Arthroderma multifidum*, *Chrysosporium*, Horse, Fungal, Sequencing and phylogenetic tree

INTRODUCTION

The Arabian horse is regarded as the oldest and most significant of all the contemporary horse breeds. Due to their extensive, distinctive, and colorful history, Arabian horses are highly economically and socially substantial. Therefore, extra care and effort are needed to prevent and control infectious diseases that could harm people's health and financial worth. Some studies evaluated the effectiveness of multiple diagnostic assays for identifying dermatophytes (Aboul-Ella et al. 2020) and those affecting Arabian horses (El-Yazeed et al. 2013; Hend et al. 2017). In contrast, other studies reported the possible risk factors of dermatophytosis in Arabian horses from Egypt (Brien et al. 2005; Buée et al. 2009). According to Chollet et al. (2015), zoophilic dermatophytes are among the most prevalent zoonotic diseases. They are typically spread by direct animal-to-human contact via fungi found on desquamate

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skin and hair. Most soil fungi are parasites of keratinized tissues and can potentially cause dermatophytosis in both humans and animals (Kaul et al. 2013). According to Cai et al. (2016), dermatophytosis is a common superficial fungal infection that has implications for public and veterinary health. However, the prevalence of this infection varies greatly depending on geographic location and other epidemiological factors like temperature, humidity, pH, climate, light exposure, and the amount of organic matter in the soil (Cafarchia et al. 2013; Cai et al. 2016). The conidial morphology of Dermatophytes is used to classify them into genera *Microsporum*, *Trichophyton*, *Epidermophyton* (in their anamorphic phase), and *Arthroderma* (in their telomorphic phase). Based on their ecological characteristics, dermatophytes are categorized as anthropophilic, geophilic, or zoophilic species. Among dermatophytes, *Arthroderma* is the most diverse genus (Hainsworth et al. 2021). When it was initially found in 1963, it was separated from the soil of rabbit hair or holes (Chabasse et al. 1989). In the past, *Arthroderma sp*. pathogenicity tests revealed a fungal infection and survival in mice's peritoneal cavity and guinea pigs' skin. However, it was unclear how pathogenic the organism was. According to Yamaguchi (2019), there is no proof of *Arthroderma sp*. infection in humans. In this study, we characterized the genome of *Arthroderma sp*. causing dermatophytosis in horses and reported a case of chronic skin fungal infection. Common soil saprobes, or members of the *Chrysosporium* genus, are primarily keratinophilic fungi that break down decomposing keratinous substrates. Only a few instances of deep infection in horses have been reported, most of which are hard to assess. For this reason, this study explores the possibility of *Chrysosporium* presence in horse farms and confirmed it using genetic sequencing and polymerase chain reaction (PCR) technology (Chen et al. 2023).

The objectives of the study are: i) To identify various fungi that are present in Egyptian Arabian horses, ii) to isolate the fungi, identify their morphology, confirm their identity using a polymerase chain reaction, and examine their genetic sequence, and iii) to analyze the genetic sequence of these fungi, studying the extent of variation amongst various species and creating a phylogenetic tree.

MATERIALS AND METHODS

Ethical approval

The experimental design was approved by the Animal Ethics Committee of the Institutional Animal Care and Use Committee (ARC-IACUC) Agricultural Research Center, Cairo, Egypt. IACUC protocol number: ARC-AHRI-95-24.

Sample collection

50 Samples were collected from infected Egyptian horses as hair plucked from the periphery of active, non-medicated lesions (Stephen 2005). Disposable gloves were used before collecting samples due to the zoonotic risk. The area was wiped with 70% isopropyl alcohol to reduce contamination of the sample. It was left to dry then a few hairs were plucked and stored in a sterile envelope (Stephen 2005).

Fungal isolation

On Sabouraud dextrose agar media supplemented with chloramphenicol and cycloheximide, each sample of horse hair roots were grown in triplicate. The cultures were kept in an incubator at 37°C for four weeks, with daily check for fungal growth starting on the third day. To remove impurities, the colonies underwent multiple subcultures and a microscope inspection. The separated fungal colonies were identified morphologically by examining their macroscopic features (growth zones, aerial and submerged hyphae, texture, color, and diffusible pigments) and microscopic traits using the slide culture technique after staining with lactophenol cotton blue.

DNA extraction of fungal cells

Genomic DNA Isolation Kit Nucleo-pore gDNA is fungal (NP-7006D); (Genetix Biotech Asia pvt ltd). The extraction process was carried out in accordance with the manufacturer's guidelines. To reduce the chance of specimen contamination, DNA extraction was done in separate rooms with equipment assigned to each location. Finally, DNA purity and concentration were detected by SPECTRO star Nano. The purified DNA was stored at 4ºC until tested.

PCR amplification and gel electrophoresis

Employed Cosmo PCR Red Master Mix (Catalog No. W1020300x, Willow Fort, UK). The mix contains the Cosmo DNA polymerase enzyme, as well as all the necessary components required for a successful PCR. MgCl2, dNTPs, storage buffer, and Cosmo Taq DNA polymerase are the ingredients of the Cosmo PCR Master Mix. According to White et al. (1990), a set of fungal primers were used for amplification with the universal primers. For every sample, the following reagents are needed: 15µL of Cosmo master mix, 2µL of forward primer, 2µL of reverse primer and 1µL from nuclease-free water. Finally, add the 5µL DNA extracted from the sample and display (Table 1). Products for amplification Agarose gel electrophoresis were used to examine the PCR, and ethidium bromide staining and a gel documentation system were used to visualize the results. The combination of amplicon and gel loading buffer (50% glycerol/0.1M EDTA, pH 8.0/1% SDS/0.1% bromophenol blue/0.0% xylene cyanole) was loaded into 1.5% agarose in 1x TBE (89mM tris/89mM boric acid/2mM EDTA, pH 8.0) The size standard was a 100bp ladder (Gibco, BRL).

Sequencing and phylogenetics

The BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher, USA) was used to sequence the DNA templates using Sanger dideoxynucleotide sequencing following PCR product purification, in accordance with the manufacturer's instructions. Reverse primers at 3.2 p.mol., were used. Centri-SepTM Spin Columns (Thermo Fisher, USA) were used to purify the sequencing products, and injection was carried out on capillary electrophoresis systems 3500 Genetic analyzers (Applied Biosystems, USA). BioEdit Sequence Alignment Editor 7.2.5 (Hall 1999) was used to prepare multiple sequence alignments of proteins. Using MEGA5.2 software, a midpoint rooted maximum likelihood phylogenetic tree was created. The tree was then confirmed using 1000 bootstrap replicates

Table 1: Amplification and cycling conditions used in this study.

Primer name	Primer sequence		Amplified segment (bp)
ST1 (Forward)	5`-TCC GTA GGT GAA CCT GCG G-3`		550 to 610bp
ST4 (Reverse)	5`-TCC TCC GCT TAT TGA TAT GC-3`		
PCR Amplification (35 cycles)			
Denaturation	Annealing	Extension	Finally, one cycle
95°C / 2 minutes	55°C / 20 seconds	72°C /1 minute.	72°C /10 minute

(Kumar et al. 2016). The matrix-based Jones–Taylor–Thornton (JTT) model is necessary for this tree to function (Jones et al. 1992). Using the Maximum Likelihood method built into the MEGA11 software, phylogenetic trees were inferred, and bootstrapping more than a thousand replicates were used to estimate the topology (Saitou and Nei 1987; Tamura et al. 2021).

RESULTS

At a government station for breeding Arabian horses, 50 Arabian horses exhibiting cutaneous lesions consistent with dermatophytosis were the source of the hair sample isolates. Fungal colonies appeared two weeks after culture, according to the culture's initial results, isolation was carried out for a preliminary microscopic inspection. A negative culture was observed for 22 samples. However, the polymerase chain reaction method showed that 24 amplification-positive samples were present out of 28 positive cultures (Table 2).

Table 2: Fungal culture of equine hair samples.

Total sample	Positive Fungal Culture	Negative Fungal Culture
(50) samples	28 Samples (56%)	22 samples (44%)
Results of positive and negative PCR amplification		
Total culture positive for fungi (28 samples)	Amplification 24 Samples	Non-Amplification 4 samples

Microscopic analysis

Direct microscopic identification of stained smears of colonial scrape material is the fastest way to diagnose presumptively. Two types of fungi were diagnosed in the equine hair samples. The first type of fungi was identified as *Arthroderma sp*., represented in the hair samples with a percentage of 34%. The colony color of this isolate on agar was white at first and gradually turned yellow a week later, the same as the color of the reverse colony. Powdery or granular colonies with flat or radial grooves were observed. For microscopy, the conidia were pear-shaped, nearly spherical or oval. The second type of fungi was *Chrysosporium sp.*, represented in the hair samples with a percentage of 22%. The microscopic appearance of *Chrysosporium sp*. in slide culture preparations shows aleurioconidia borne at the tips of short, typically curved stalks (curved arrow) or sessile (straight arrow). The isolates were resistant to cycloheximide, as judged by its equivalent growth on Mycosel medium (BBL). The microscopic morphology was examined in slide culture preparations. Conidia (aleurioconidia) was formed at the ends of short stalks that arise at an acute angle and are often slightly curved.

Amplification and sequences analysis

Using ITS1 and ITS4 primers, purified genomic DNA was prepared for PCR amplification of the ITS regions, producing fragments with lengths varying from 531 to 580bp (Fig. 1). The outcome was 24 samples (16 samples of *Arthroderma sp*. and 8 samples of *Chrysosporium sp*.) that identified by microscopic inspection for amplification of the same two isolated species. Subsequently, the purified DNA fragments underwent nucleotide sequencing using distinct primer sets. Following editing and analysis, the sequences were submitted to GenBank, where Table 3 displays the obtained accession numbers. It is common practice to examine fungal variability at the species level using the ITS regions of rDNA (Bonito et al. 2010; Mohankumar et al. 2010).

Table 3: Different isolation fungal of PCR amplification with sequence analysis.

Sample Number	Fungal isolates	Organism	Strain (*n*)[a]
Sample 6			PP085493
Sample 11			PP085494
Sample 30			PP085495
Sample 35	Fungal	*Arthroderma*	PP085496
Sample 39	*Arthroderma*	*multifidum*	PP086051
Sample 49			PP086052
Sample 15	Fungal	*Chrysosporium*	PP085497
Sample 26	*Chrysosporium*	*zonatum*	PP086050

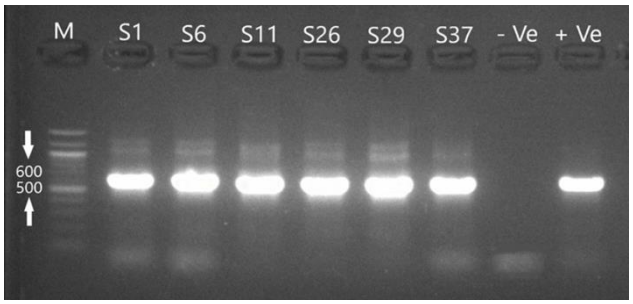

Fig. 1: Agarose gel electrophoresis of the ITS regions amplified using ITS1 and ITS4 PCR primers. The gel shows (respectively from left to right) Gene ladder 100 bp DNA Ladder, lane (1, 2 and 3) samples (S1, S6 and S11) as (*Arthroderma multifidum* with 580 bp), lane (4, 5 and 6) samples (S26, S29 and S37) as (*Chrysosporium zonatum* with 580 bp), lane (7) negative control and lane (8) positive control.

The present study's sequences' database matching results verified that the two isolates under examination belonged to two distinct genera (Table 3). For every isolate, the amplified sequence was aligned using BLASTn with the sequences of closely related species belonging to the same taxonomic group in the GenBank database. The study's findings demonstrated that all isolates' sequences had (100%) similarity with the sequences of numerous species that had previously been identified as *Chrysosporium zonatum* (PP085497 and PP086050) and *Arthroderma multifidum* (PP085493, PP085494, PP085495, PP085496, PP086051 and PP086052). Phylogenetic Tree: The fungal isolates' phylogenetic trees were created using the Mega 5.2 program. Sequences from

the GenBank are displayed without a marker, while sequences from this study are indicated in red.

Concatenated alignment phylogenies and supertree phylogenies are generally highly congruent (Fig. 2). *Arthroderma multifidum* and *Chrysosporium zonatum's* internal transcribed spacer (ITS) rDNA sequences yielded a similar tree, with a bootstrap of 1000 replications across all, the phylogenetic connections between various isolates (Fig. 2).

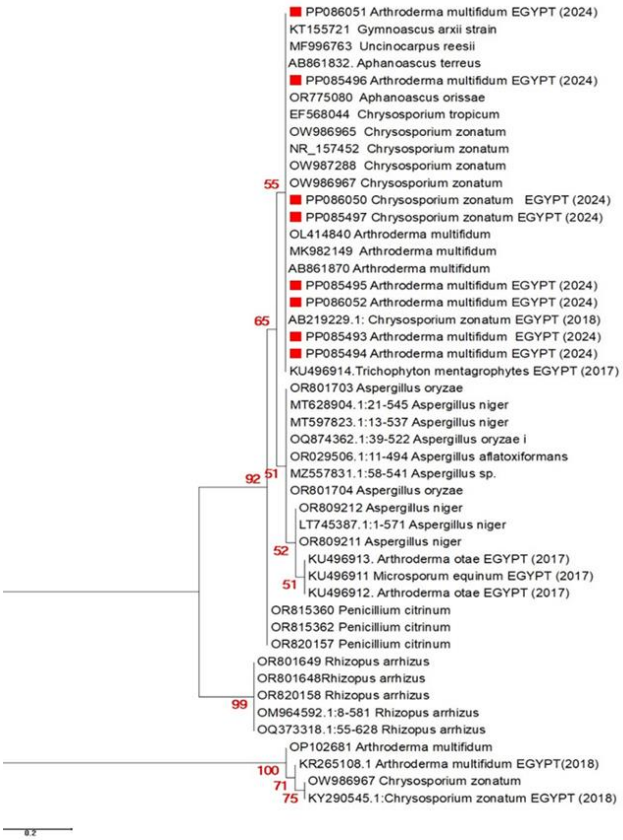

Fig. 2: Phylogenetic tree based on ITS region of rDNA gene sequences for fungi obtained in this study with their reference strains

DISCUSSION

As one of the most significant fungal skin diseases that affect horses is dermatophytosis that must be diagnosed as soon as possible to develop a successful treatment plan. Dermatophytes only need to be identified by genus to be treated appropriately; however, species identification is crucial for epidemiologic purposes to identify the source of the infection and implement preventive measures (Hend et al. 2017). Holtgrew-Bohling (2016) states that dermatophytosis is a common fungal infection of the skin and hair's superficial layers. A class of fungi known as dermatophytes invades and breaks down keratinized tissues, such as skin, hair, nails, and feathers (Weitzman and Summerbell 1995). These fungi are members of the *Arthrodermataceae* family, the *Onygenales* order, the *Eurotiomycetes* class, and the *Ascomycota* phylum (De Hoog et al. 2017; Segal and Elad 2021). Currently, dermatophytes are classified into seven recognized genera: *Arthroderma*, *Paraphyton*, *Lophophyton*, *Nannizzia*, *Microsporum*, *Trichophyton*, and *Epidermophyton* (De Hoog et al. 2017). For equine illnesses, a precise diagnosis is essential for applying suitable treatments. Since the clinical presentations of many dermatophytosis forms overlap with those of other skin conditions, it can be challenging to differentiate them clinically (Garg et al. 2009). The fungi chosen for the analysis were first categorized as dermatophytes species based on their macroscopic and microscopic characteristics (Domsch et al. 2007). *Arthroderma sp.* and *Chrysosporium sp*. were the species of fungi that were identified by the PCR reaction (Bohacz et al. 2020). These findings, which came from several kinds of farms in Egypt, supported and confirmed earlier studies and research on the existence of these two types of fungi in these various locations. To investigate genetic alterations and precisely identify the two species, it was preferable to perform genetic sequencing (Chen et al. 2023).

On the other hand, in the process of applying the gel electrophoresis method, in which the ITS region lengths may be similar, it is highly probable that their sequences differ and may potentially be differentiated by single-strand conformational polymorphism (SSCP) (Turenne et al. 1999). All sequences results of ITS1-5.8S-ITS4 regions showed similarity to all isolates of amplified sequences subjected to BLASTn alignment with sequences of those closely related species in the same taxa in the GenBank database. Based on nucleotide sequencing, the fungi were identified as *Arthroderma multifidum* (6 samples) and *Chrysosporium zonatum* (2 samples). The phylogenetic tree used in this study analysis of GenBank's accession numbers (highlighted in red) matched precisely. A bootstrap of 1000 replications for each fungal isolate was used to test phylogeny (Hend et al. 2017).

Additionally, a phylogenetic tree based on previous studies of Egyptian horses that isolated three additional types of *Arthroderma* (*KU496911-micosporum, KU496912-Arthroderma otae, and KU496913-Arthroderma otae*) was included. However, it also contained some soil-related research from Egypt, wherein *Arthroderma multifidum* (KR265108) and *Chrysosporium zonatum* (ky290545) were isolated. The phylogenetic tree for the same isolate showed significant differences between the study's findings (Hend et al. 2017). Compared to *Arthroderma multifidum, Chrysosporium zonatum* have fewer simple records. This is because they are more common in the soil and surroundings of stables.

Given that the horse participated in numerous outdoor activities in the farmer's yard, it is possible that the infection was contracted through exposure to airborne germs (Sigler et al. 1999). While species of *Chrysosporium* and Dermatophytes are free-living in their surroundings, they can infect human and other animals in specific situations. These fungi are filamentous, septate, hyaline molds that can produce spores or conidia and are primarily made of mycelium (Samanta 2015). Since the fungus can cause infections to linger in the environment for up to 12 months so all areas that come in contact with the horse must be thoroughly cleaned and disinfected to stop the infection from spreading (Holtgrew-Bohling 2016). The spread of fungi can also cause material harm because Arabian horses are valued economically in Egypt and the Arab world (Swain 2003). However, one of the most crucial measures

that needs to be considered is cleaning and disinfecting all surfaces and equipment that have come into contact with infected horses (Dillon 2012).

Conclusion

The accessibility of genomic data for *Arthroderma multifidum* and *Chrysosporium zonatum*, combined with an extensive list of previously recognized molecular tools, would enable more comprehensive research aimed at comprehending dermatophytes.

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Author's contribution

Doaa D. Khalaf, Engy Farahat and Mohamed I. Abdallah designed the plan for the work. Nahla Hussien Abou El Ela and Sara M. Elnomrosy reviewed and drafted the manuscript with Mohamed I. Abdallah. They all contributed to this PCR laboratory work with sequencing analysis steps and phylogenetic tree. Doaa D. Khalaf and Engy Farahat, cultured and isolated the fungal strains, analyzed data, and also contributed to the paper publication. Hussein A. Abuelhag contributed to the study design and data analysis with final revision.

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