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Molecular Identification of Fungal Pathogens causing Feather Infection in Parrots Suffering from the Psittacine Beak and Feather Disease (PBFD) Virus

Mohamed I AbdAllah[1]*, Sara M Elnomrosy[1], Nahla Hussien AbouEl Ela[1,5], Engy Farahat[2], Doaa D Khalaf[2], Khadijah A Altammar[3] and Rasha MH Sayed-ElAhl[4]

[1]Genome Research Unit, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Cairo, Egypt
[2]Department of Microbiology and Immunology, National Research Centre, Dokki, Cairo, Egypt
[3]Department of Biology, College of Science, University of Hafr Al Batin, PO Box 1083, Hafr Al Batin 31991, Saudi Arabia
[4]Department of Mycology, Animal Health Research Institute (AHRI), Agriculture Research Center (ARC), Cairo, Egypt
[5]Department of Veterinary Science, College of Agriculture, Food, and Environment, University of Kentucky, Lexington, KY, USA
***Corresponding author:** yja_mohamed75@yahoo.com

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ABSTRACT

Accurate identification of infectious molds from clinical specimens is crucial, as evidenced by the rising incidence of invasive fungal diseases in birds and their growing frequency. Fungal DNA was found in parrot feather samples linked to Psittacine Beak and Feather Disease (PBFD) virus infection following the detection of culture isolates. Internal transcribed spacer regions (ITS) have been employed as targets for phylogenetic research as they typically exhibit sequence variation between species, with modest change within strains of the same species. Numerous sequence variations were found when comparing ITS region sequences from reference and clinical isolates of six *Aspergillus* species, one *Rhizopus arrhizus*, and two *Penicillium* species, The addition of 5.8S rRNA gene sequences had little effect on the species as a whole due to the presence of little diversity between species in this region. Amplification, sequencing, and comparison with non-reference strain sequences in GenBank were performed on strains and clinical isolates of aspergilli and other fungi. *Aspergillus* and other species amplified ITS region had a size range of 540 to 608 base pairs (bp). A bootstrap of 1000 replications of every fungal isolate was also displayed on the phylogenetic tree, along with varying percentage rates among the fungal isolates.

Key words: Circovirus, PBFD, *Aspergillus, Rhizopus, Penicillium*, Sequencing and Phylogenetic Tree.

INTRODUCTION

All Psittacine species are susceptible to the infectious and frequently fatal Psittacine Beak and Feather Disease (PBFD), which has been identified in both wild and captive parrot populations (Alaudeen et al. 2017). The definitive identification of the etiologic agents of skin disease in companion birds has a significant role in health and immunology, however, there are insufficient studies of the normal skin and feather flora of these species (Rubinstein and Lightfoot 2014). Prior research has demonstrated that common fungal isolates from the skin and feathers of healthy parrots include *Aspergillus, Corynebacterium, Acinetobacter,* and *Staphylococcus* species. According to the few reports of bacterial and fungal skin pathogens in parrots, some of these microbes may cause skin injury or immune deficiency in order to become pathogenic (Koski 2002; Van der Eijk et al. 2019). Birds could have efficient means of dispersing microorganisms either in the wild as they can fly for long distances, or in urban settings by being in close proximity with humans and other animal's vulnerable hosts to infections. Furthermore, handling, storing and preparing game birds can expose people to keratinophilic fungi that are present on the outer contour of the feathers and are inhibited by the bird's body temperature (Pugh 1970). Some bird species function as potential carriers of pathogenic fungi in their bird nests (Korniłłowicz-Kowalska and Kitowski 2013). This study aims to assess the rate of secondary fungal infection in laboratory-confirmed cases of PBFD virus (Gugnani 2000). Because PCR-based techniques are quick, sensitive, and precise, they are now frequently used to identify a wide range of pathogens (Edel et al. 1997; O'Donnell et al. 2010; Yadav et al. 2011).

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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- ABC.AHRI-8-24.

Samples

Using sterile forceps, the feathers were collected from diseased and healthy parrots, 50 of each, and placed in a sterile envelope for storage and transferred to the lab for fungal examination. PCR was used to identify the PBFD virus. According to records, the first 50 samples of birds have PBFD virus infection, while the remaining 50 samples of birds are parrots in good health.

Detection of Psittacine Beak and Feather Disease (PBFD) virus

DNA was extracted from one hundred feather samples using the QIAGEN DNA Extraction Tissue Kits, nos. 69504 and 69506. We used Dream Taq Green PCR Master Mix (2X) (pub, no. MAN0012704) for the PCR amplification of the PBFD virus. 10µL of master mix was prepared. We added 3µL of water, 1.5µL of each primer at 20pmol (5 TTAACAACCCTACAGACGGCGA~3;5~ GGCGGAGCATCTCGCAATAAG~3, and then added 4µL of the sample's extracted DNA. The reactions involved of one cycle of initial denaturation at 95°C for 3min, then 35 cycles of denaturation at 95°C for 30s, annealing at 54.5°C for 30s, and extension at 72°C for 60s, concluding with one cycle at 72°C for 5min.

Fungal isolation and identification

Every sample was cultivated in triplicate on Sabouraud dextrose agar medium (SDA powder purchased from Oxoid). The cultures were incubated at 25°C for 5-7 days. The colonies were stained with lactophenol cotton blue and were examined under a microscope. The isolated fungal colonies were identified by analyzing their morphological macroscopic features (topography, growth rate, color, texture, diffusible pigments, exudates, growth zones, aerial and submerged hyphae), and their microscopic features using slide culture technique (Pitt and Hocking 2009).

DNA extraction of fungal cells

We used Genomic DNA extraction Kit Nucleopore gDNA (NP-7006D) as a specific kit for fugal DNA isolation. 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 fungal amplification

We used a Cosmo PCR red Master Mix kit for DNA amplification: (Willow Fort, UK, Catalog No: W1020300x). The mix contains the Cosmo DNA polymerase enzyme, and all the necessary components required for a successful PCR. Components of the Cosmo PCR Master Mix include Cosmo Taq DNA polymerase, $MgCl_2$, dNTPs and storage buffer. For amplification, the universal primers used two fungal primers as reported by White (1990). ITS 1, ~5-TCC GTA GGT GAA CCT GCG G-}3; ITS 4, ~5-TCC TCC GCT TAT TGA TAT GC-}3) are primers for the ITS region. Each sample requires the following reagents: 10µL of Cosmo master mix, 2µL of forward and reverse primers with 3µL from nuclease-free water. Added the 5µL DNA that was extracted from the sample lastly. The reaction consisted of one cycle of initial denaturation at 95°C for 2min. Followed by 35 cycles consisting of denaturation at 95°C for 15s, annealing at 52°C for 20s, and extension at 72°C for 60s. Finally, there was one cycle at 72°C for 10 min.

Gel electrophoresis

Products for amplification PCR were examined using agarose gel electrophoresis and visualized using gel documentation system and ethidium bromide staining. In 1.5% agarose in 1x TBE (89 mM tris/89 mM boric acid/2 mM EDTA, pH 8.0), 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. The size standard was a 100bp ladder (Gibco, BRL).

Sequencing and phylogenetics

A gel extraction kit (QIAquick, Qiagen) was used to cut and purify positive DNA bands with the anticipated sizes in accordance with the manufacturer's instructions. The purified products were sequenced using the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequencing reactions were then purified using the Dye EX Qiagen Purification Kit. Following purification, the sequence products were decoded using an Applied Biosystems 3500 Genetic Analyzer. Protein multiple sequence alignments were prepared using the BioEdit Sequence Alignment Editor 7.2.5 (Hall 1999). A midpoint rooted maximum likelihood phylogenetic tree was constructed using MEGA7 software, and 1000 bootstrap replicates were used to confirm the tree (Kumar et al. 2016). This tree is dependent upon the matrix-based Jones–Taylor–Thornton (JTT) model (Jones et al. 1992), as well as the tree.

RESULTS

Numerous positive samples confirm the Psittacine Beak and Feather Disease (PBFD) virus's existence. The illness is characterized by the bird's deformity, loss of feathers, and decreased activity. Every positive case of the PBFD virus has abnormalities in the beak and feathers. Because the contour feathers are abnormal, PBFD decreases the amount of feather dust produced. The colors of the feet and beak change, and some of the newly emerging feathers are colorless tiny, twisted, and highly abnormal. All tail feathers and deformed beaks are absent, and even when new feathers are added, they do not grow in. Out of all the feather samples, more than 52 samples were obtained. PBFD virus genome was amplified using PCR as a 603bp fragment (Nucleotides 178-780) with the primers as described above. The culture results showed that there were 22 positive samples from the 50 positive feather samples infected with the virus. Fungal colonies appeared 5-7 days after inoculating, and isolation was done for initial examination using a microscope. Also, in other healthy case samples, 6 fungal colonies were seen in Table 1.

Table 1: PCR analysis and fungal culture of feather scrapings from the roots of parrots

Total sample (100)	Positive Fungi Culture	Negative Fungi Culture
Group (1): disease cases 50 samples (Number 1 to 50)	22 samples (44%)	28 samples (56%)
Group (2): Healthy parrots (50 samples: Number 51 to 100)	6 samples (12 %)	44 samples (88 %)
Results of positive and negative amplification PCR of two groups		
Total culture positive for fungal (28 samples)	Amplification PCR	Non-Amplification PCR
Group (1): Positive Fungi Culture	20 Samples	2 samples (Culture result unidentified)
Group (2): Positive Fungi Culture	6 samples	---------------

Identification of fungal species by microscopic analysis

Direct microscopic examination of stained smears of colonial scrape material is the fastest means of making a provisional diagnosis. Three types of fungi were diagnosed in the feather samples of the first group infected with the PBFD virus, the first type of fungi *Aspergillus* species was observed in the infected group, they are represented in the following samples feathers (2, 3, 6, 7, 12, 13, 14, 21 and 44). The analysis identified four distinct *Aspergillus* species types: *Aspergillus nidulans*: the short, columnar, and biseriate heads of the colony, conidiophores are typically smooth-walled, short, and brownish in color; conidia have rough walls and a globose shape. Colonies of *Aspergillus niger* are initially fuzzy, ranging in color from white to yellow to dark brown to black, the reverse is yellow to white, the phialides are biseriate and organized in two rows, spanning the entire vesicle to form a radiating head; the conidiophore varies in length. *Aspergillus flavus*: the silky, yellow-green to brown colonies, the conidiophores have uneven lengths and rough surfaces, and the reverse was golden to reddish brown. *Aspergillus terreus*: a cinnamon-brown flat colonies with a velvety to powdery texture; Smooth-walled conidiophores bearing typical columnar and biseriate conidial heads which produce elliptical conidia. *Penicillium citrinum* is the second type of mold; its colonies generate septate, hyaline (clear, non-pigmented) hyphae and conidiophores with smooth walls having long stipes observed. Also, that set is apart from other species include spherical conidia that are produced in well-defined chains and medullae that are longer than phialides. These characteristics can be seen in the feathers of the following samples: 5, 11, 23, 29, 31, 38, and 41. *Penicillium citrinum* represents 14% of the infected group, while *Penicillium citrinum* and *Penicillium dipodomyicola* represent 4% of the healthy group in samples 59 and 72 (Fig. 1 and 2). *Rhizopus arrhizus* is the third type; the colonies grow quickly, exhibit smooth walls, are non-septate, simple, or branched, and arise from stolons opposite rhizoids, usually in groups of three or more. Samples 16, 20, 27, 34, 36, and 37, exhibited these colonies. The *Rhizopus arrhizus* represent 12% of the infected group, and 8% of the healthy group in samples 51, 53, 77 and 87 (Fig. 1 and 2).

Internal Transcribed Spacer (ITS) region of the nuclear ribosome as a universal DNA barcode marker for fungal

All positive cultures DNA were amplified by PCR using the universal fungal-specific primer pair ITS1 and ITS4. After ethidium bromide staining and electrophoresis,

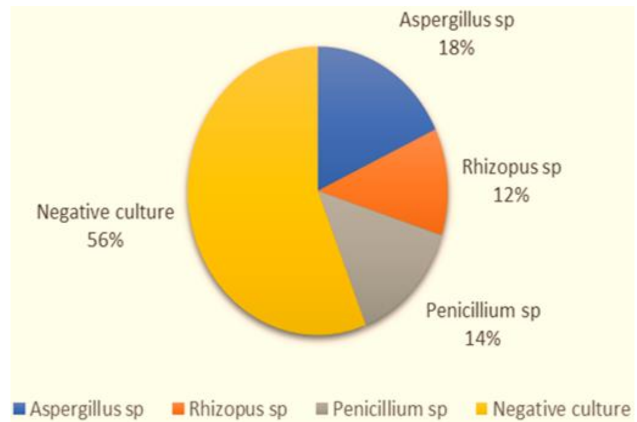

Fig. 1: The graph shows the different percentages of the three types of fungi under study in the group infected with the PBFD virus.

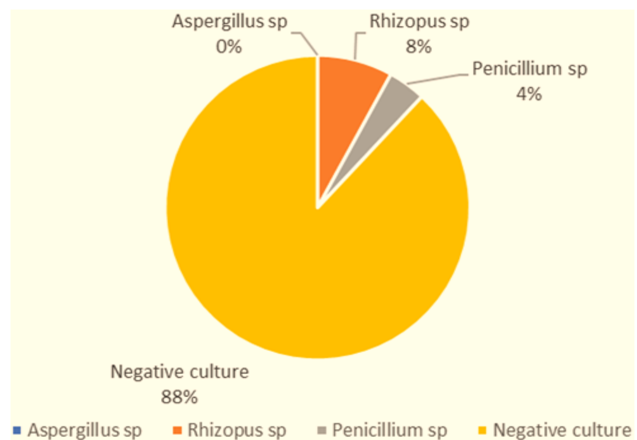

Fig. 2: The graph shows the different percentages of the three types of fungi under study in the non -infected group with the PBFD virus.

amplicons were found in agarose gels. DNA from every type of fungal species used to create the DNA ladder. In eukaryotes, the highly amplified nuclear ribosomal Internal Transcribed Spacer (ITS) region is made up of the ITS1 spacer, which is located between 18S and 5.8S rRNA genes, and the ITS2 spacer, which is located between 5.8S and 28S rRNA genes. Small-subunit (SSU) nr RNA is present in the 18S and is amplified in numerous fungal phylum, classes, and families. However, the 28S rRNA genes are amplifications of genus and family. Primers (arrows) that are forward and reverse can be used to amplify the entire. The molecular weight of various isolates was examined using rDNA amplification in PCR as shown in Fig. 3 and 4.

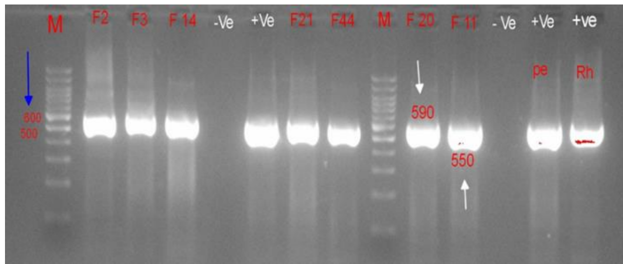

Fig. 3: Agarose gel electrophoresis of DNA amplified fungal (ITS region) by PCR reaction. lane (1) M: (100) bp DNA molecular size marker, Lane (2): feather 2 (Aspergillus niger, 599 bp); Lane (3): feather 3 (Aspergillus oryzae, 596 bp); Lane (4): feather 14 (Aspergillus flavus, 595 bp); Lanes (5), (6), and (7): feather 21 (Aspergillus sydowii, 598 bp). lane (8) M: (100) bp DNA molecular size marker, lane (9) feather 20 as (Rhizopus arrhizus with 590 bp), lane (10) feather 11 as (Penicillium citrinum with 545 bp) lane (11) negative control, lane (12) positive control Penicillium citrinum, and lane (13) positive control Rhizopus arrhizus. Blue arow detection region between 500 and 600 bp.

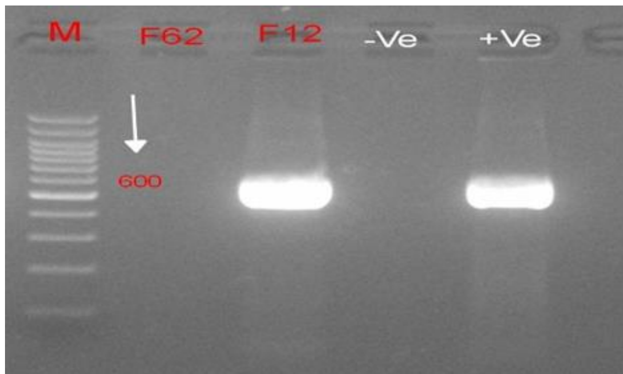

Fig. 4: Agarose gel electrophoresis of DNA amplified fungal (ITS region) by PCR reaction. lane (1) M: (100) bp DNA molecular size marker, lane (2) feather 62 as (negative fungal), lane (3) feather 12 as (Aspergillus terreus with 608 bp), lane (4) negative control, lane (5) positive control.

Gene sequences of fungal isolates

Analyzing the alignments revealed that all isolates had contiguous sequences of the ITS1-5.8S-ITS2 regions. The amplified sequence was aligned using BLASTn with the sequences of closely related species belonging to the same taxonomic group in the GenBank database. *A. oryzae* OR801703, *A. oryzae* OR801704, *A. niger* OR809211, *A. niger* OR809212, *A. terreus* OR819417, *A. sydowii* OR819418, *A. nidulans* OR820156, and *A. flavus* OR819416 are among the many species previously identified as all Aspergillus isolation species. The results of comparisons between referenced strain sequences and clinical isolates of the same Penicillium species. For *Penicillium dipodomyicola* (OR819419) and *Penicillium citrinum* (OR815360, OR815361, OR815362 and OR8120157), there was simple intraspecies variation. The total sequence similarity between the clinical isolates of the same species and the referenced Penicillium strains was more than 100% when considering the length of the amplified ITS region. All isolates in this investigation were identified as *Rhizopus arrhizus* (OR801648, OR801649, and OR820158), based on the highest aligned bit score of Rhizopus species addressed in the listed BLAST search (Table 2).

Table 2: Sequence analysis of different fungal isolation with an accession number of Gen Bank

Number of positive samples	Fungal isolation by culture and microscopic analysis	Typing of fungal-isolation by sequence analysis	The accession number of GenBank
Feather (16)	Rhizopus species	*Rhizopus arrhizus*	OR801648
Feather (20)		*Rhizopus arrhizus*	OR801649
Feather (5)		*Penicillium citrinum*	OR815360
Feather (11)	Penicillium species	*Penicillium citrinum*	OR815361
Feather (19)		*Penicillium citrinum*	OR815362
Feather (3)		*Aspergillus oryzae*	OR801703
Feather (13)		*Aspergillus oryzae*	OR801704
Feather (2)		*Aspergillus niger*	OR809211
Feather (7)		*Aspergillus niger*	OR809212
Feather (12)	Aspergillus species	*Aspergillus terreus*	OR819417
Feather (21)		*Aspergillus sydowii*	OR819418
Feather (44)		*Aspergillus nidulans*	OR820156
Feather (14)		*Aspergillus flavus*	OR819416
Different isolation fungal of PCR Amplification with healthy cases in feathers by sequence analysis			
Feather (51)	Rhizopus species	*Rhizopus arrhizus*	OR820158
Feather (59)	Penicillium species	*Penicillium citrinum*	OR820157
Feather (72)		*Penicillium dipodomyicola*	OR819419

Phylogenetic tree

Phylogenetic trees of the fungal isolates were created using the Mega 5.2 program. Sequences from the GenBank are displayed in black without a marker, while sequences from this study are indicated in purple. A bootstrap of 1000 replications of every isolated fungal sample was used to assess phylogeny. Concatenated alignment phylogenies and super tree phylogenies are highly congruent in general. The tree in Fig. 5 was created using the internal transcribed spacer (ITS) rDNA sequences of *Aspergillus* species. A bootstrap of 1000 replications was used to ensure their similarity. The evolutionary connections between *Penicillium dipodomyicola* (OR819419) and *Rhizopus* species (Fig. 6) exhibit similarity in their ITS rDNA sequences, with a bootstrap of 1000 replications for each species, which demonstrated resemblance to the other types of *Penicillium,* such as: *Penicillium griseofulvum* (KX056234-OR810001-MH865646); *Penicillium chrysogenum* (KU878095) and other species (OL685263-FJ623269- KX588061).

DISCUSSION

Previous studies have shown the spread of the PBFD virus in many regions around the world((Heath et al. 2004; Deborah et al. 2016) suggested that the PBFD virus may induce long-term immunological suppression. One of the most common diseases found in captive parrots is feather damaging behavior, also known as feather picking (Tang et al. 2020). Circovirus, on the other hand, targets the Thymus and Bursa of Fabricius specifically, inhibiting the production of lymphocytes and seriously weakening the bird's immune system. The immunosuppression is more severe in younger infected birds. In the first three to six weeks of life, birds develop their antibody diversity in the Bursa of Fabricius; those infected before they have developed any immune function will never build a strong enough immune system. The PBFD virus causes these

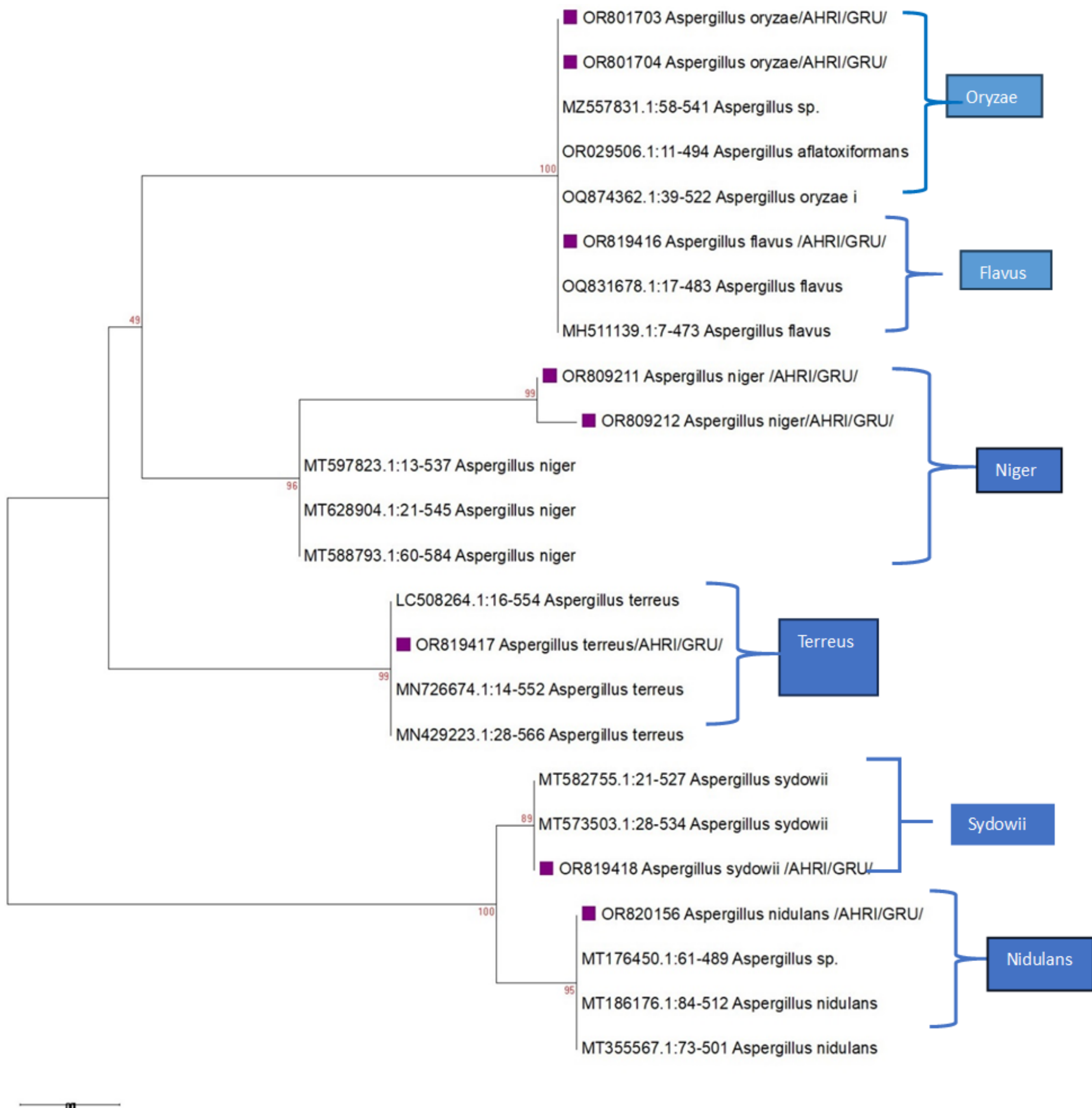

Fig. 5: *Phylogenetic tree of different Aspergillus species compared with reference Aspergillus* strains.

birds' immune systems to be suppressed, which can lead to a variety of secondary infections. Often, a veterinarian will only test for PBFD if a strange infection is noticed (Michael and Pyne 2005).

The immunosuppression developed in the course of PBFD viral infection predisposes parrots to variable fungal infections (Brett et al. 2017). *Aspergillus* species as *A. flavus, A. terreus, A. niger, A. nidulans, A. sydowii,* and *A. oryzae,* were among the various fungal species found in the cultures and examinations (Denning 1998). *A. flavus* isolation in parrots has been reported by Churria et al. (2012). Vertebrate epithelial cells produce keratins which are the main components of a horny epidermis layer and its byproducts, like feathers, are proteins (Asquith 1977; Timar-Balazsy and Eastop 1998; Hyo-Min et al. 2017).

PBFD virus can cause immune deficiency, deformities, and decomposition of feathers and beaks, which is a suitable medium for these fungi (Heath et al. 2004), especially the *Aspergillus* and *Penicillium* genera (Barbara 2009). However, in the healthy group there were only two types of fungi (*Rhizopus arrhizus* and *Penicillium*). This is due to the possibility of the presence of mold in the fruit that the parrots feed on, especially apples (Fiori et al. 2008; Holb and Scherm 2008; Gryganskyi et al. 2018). The presence of these two fungi in both groups could be explained as they are linked to food, but they become more opportunistic in the presence of the BPFD virus. However, the *Aspergillus* species were limited to the group infected with the BPFD virus only. *Rhizopus*-associated mucormycosis despite being less common than fungal infections brought on by *Aspergillus species* ascomycete, however, it is considered a pathogen in cases of immunodeficiency (Muszewska et al. 2011; Tribble et al. 2011).

Genetic analysis is valuable in detecting the types of isolated fungi and studying the differences and variations between the same type of fungi. Therefore, we

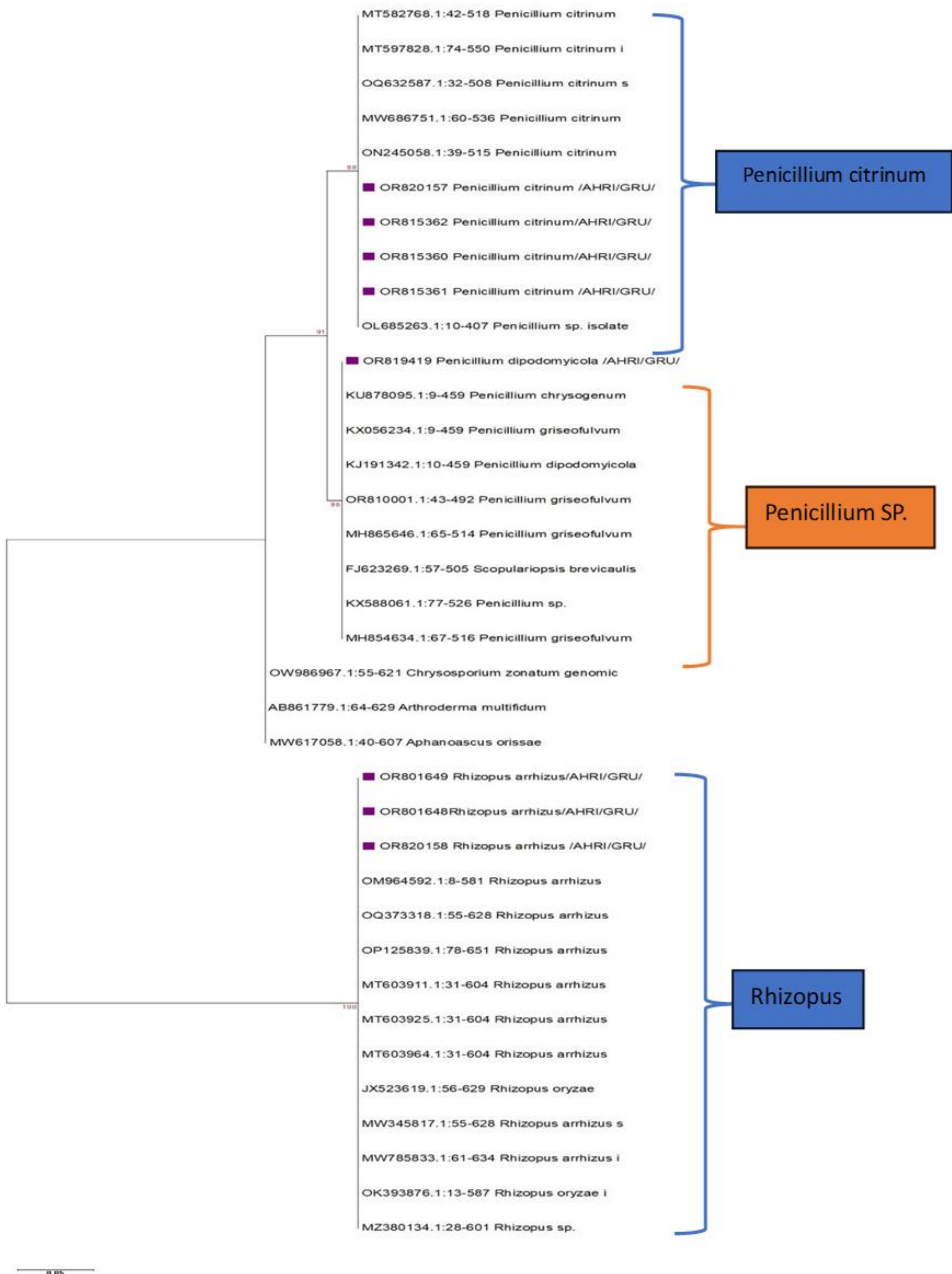

Fig. 6: Phylogenetic tree of Rhizopus arrhizus and different species of Penicillium compared with reference strains.

used ITS amplified followed by sequencing and tree-building techniques to confirm the phenotypic identification and evaluate evolutionary relationships (Lücking et al. 2020).

Six clinically relevant strains of *Aspergillus* and one *Rhizopus arrhizus* were amplified in the ITS 1–5.8S–ITS 4 regions, yielding PCR products with a size range of 565–613 base pairs (Travis et al. 2000). On the other hand, *Penicillium* species are products ranging in size from 540 to 550 (Tiwari et al. 2011; Wita et al. 2021).

According to previous studies (Brien et al. 2005; Buée et al. 2009), all isolates of amplified sequences that underwent BLASTn alignment with sequences of those closely related species in the same taxa in the GenBank database displayed similarity to all results sequences of ITS1-5.8S-ITS4 regions. According to Geiser et al. (2007), *Aspergillus* possesses ITS sequences that are identical in multiple complexes of critical mycotoxigenic. In species-rich *Pezizomycotina* genera with shorter amplicons, like the economically significant genera *Penicillium*, ITS sequences shared between different species have already been reported (Skouboe et al. 1999).

Different fungal groups have different delineations for their species, which are frequently impacted by a lack of sample availability and in-depth biological understanding. This impact is evident when ITS distances are compared between phyla, subphyla, and species (Taylor et al. 2000; Hoffmann et al. 2011). The rates of secondary fungal infections were higher in positive cases PBFD virus than in healthy cases (Gugnani 2000).

Conclusion

This study demonstrates the relationship between the presence of some types of opportunistic fungi and the presence of the BPFD virus in the feathers and skin of birds, which specialized veterinary authorities should consider when treating birds.

Acknowledgement

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Conflict of interest

The authors have declared no conflict of interest.

Author's Contributions

Mohamed I. Abdallah designed the plan for the work. Nahla Hussien AbouEl 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, analyzed data, and also contributed to the paper publication. Khadijah A. Altammar analyzed the data and reviewed the manuscript. Rasha M.H. Sayed contributed to the study design and data analysis with final revision.

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