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Original Article

Evolutionary Relationship and the Sequence Similarities among Different Fungal Species Infecting Birds Captured from Different Areas in Denmark

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Abstract

Fungal diseases are the common cause of death in wild animals and birds of prey. This study was designed to investigate the development of fungal infections among wild birds in Denmark. In this study, fungal samples were isolated from such sources as Barn swallows' feathers, White stork, and birds of prey. The fungal species were isolated by direct culture of feathers on SD Agar with chloramphenicol and incubated at 28±2°C. The fungal genomic DNA was isolated from each species, PCR reaction was performed, and the resulting fragments of the 18S rRNA DNA were sequenced and used for identification. A comparison between the resulting fragments was made to find out the percentage of similarity among the different fungal species. The multiple sequence alignment showed percentages of similarities ranging from 39% to 99%. To sum up, the 18S rRNA DNA sequence has been evolved dramatically even within the same species, while still conserved in others. It is a useful tool to be used for the identification of fungal species as it reduces time. Moreover, according to the results, there were no comprehensive high homology percentages among the species infecting the same bird.

Keywords: evolutionary, sequence, similarities, Denmark

1. Introduction

Fungal infections caused by fungi, as well as eukaryotic and spore-producing microorganisms, are ranked among the most common infectious diseases in wild birds, as well as prey and predator birds. Fungal infections are commonly considered the most cause of death in captive and recently captured free-ranging birds of prey. These microorganisms have been considered the most common nontraumatic disease-affecting wild-life birds and mammals (1).

Fungi have been present in wild and domestic animals. Most of these microorganisms are mutualistic or commensal (2). Moreover, they have played a biological key role in predator-prey interactions. In a study conducted by Møller, Erritzøe (3), the recorded data revealed that the size of uropygial glands, the uropygial gland of birds producing chemical substances with antifungal and antimicrobial properties which have shown an increase in the size of the gland in the infected birds, is strongly related to the chance of survival from being hunted by predators, such as goshawk *Accipiter gentilis*. Microorganisms can have a powerful drawback influence on their hosts' health and fitness. Fungi and bacteria are a cause of disease and mortality in wild birds. Animals have expanded many mechanisms of defense to get rid of such infections (4).

Microorganisms, fungi, and bacteria are able to decrease the feathers' quality by degradation of the feather in a significant way, thereby decreasing the prey's flight ability (5, 6). In a study conducted by Al

Rubaiee, Al Murayati (7), it was shown that the probability of having damaged feathers increased with the number of fungal colonies, and in particular, the abundance of *Myceliophthora verrucosa* and *Schizophyllum spp.* was positively related to the probability of having damaged feathers.

Genome sequencing and analysis have significant importance to uncover prominent sequence similarities among different fungi species, and these analytic results can be used to infer homology among different fungi species (8). The evaluation of sequence similarity is critical, and it is considered the first step in assessing genomic sequences; moreover, it has a wide range of applications to discover the evolutionary link among different fungi species. This is based on the idea that two species with similar sequences are related in terms of evolution (9). The most common fungal phylogenetic markers are the 28S and 18S rRNA gene sequences (10). Despite the fact that the 28S rRNA gene sometimes resolves to a lower taxonomic level, the majority of publically available sequence data is for the 18S rRNA gene (11).

The current study was designed to investigate the percentage of homology among the microorganisms infecting the same species of birds and whether there is a homology among the same genus of the infectious organisms isolated from the birds in this work through comparing the DNA sequence of their isolated 18S rRNA. This could give hints of the possibility of an evolutionary relationship among these infectious microorganisms hosting the same bird species and how far is the spacing percentage of homology of the same species that infect the other species of birds in this study.

2. Materials and Methods

2.1. Study Location

This study was performed in the rural areas across Denmark in 2020.

2.2. Fungal Isolation

Feathers from the White stork, Barn swallow, as well as prey and predator, was collected according to a study by (7), Al Rubaiee, Al Murayati (12), (13). The fungal species were isolated by direct culture of feathers on SD Agar with chloramphenicol and incubated at 28±2ºC. On the third day of fungal growth, cultures were incubated with daily examination for four weeks. SD Agar with chloramphenicol was used to culture the samples on a daily basis. The cultures were incubated for two weeks extra for identification purposes.

2.3. Molecular Identification

For the identification purposes, the 18S rRNA DNA fragments were used by picking up all amounts of mycelium to the growing plate, suspending in 200 µL in an Eppendorf tube, and storing at -20°C for more processing. The PowerSoil® DNA Isolation Kit (MO BIO) was then utilized to isolate the fungal genomic DNA according to the kit instructions. Subsequently, the purified DNA was eluted. The PCR was conducted using a reverse primer (5'-CTCTCAATCTGTCAATCCTTATT -3′), 0.5 µl, and forward primer (5'-CGA ATC GCA TGG CCT TG-3′), 1 µl fungal DNA, 0.25 µlGoTaq® Flexi DNA polymerase and completed to a 50 µl distilled water.

To prepare the PCR products, 1% agarose gel electrophoresis was used in 0.5 X Tris-Acetate-EDTA to quantify the fragments' sizes, with a running time of 30 min. To stain the gel, Gel Red (BIOTIUM) was utilized for 30 min using the photo documentation system by imaging under a UV lamp.

2.4. DNA Sequencing and Comparison

In order to perform DNA sequencing for further comparison, the PCR products were then sent to Beckman Coulter Genomics, Takeley, Essex, UK. The NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to handle the sequencing results. DNA sequence comparison was carried out by multiple sequence alignment using EMBOSS Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.5. Level of Homology

In this study, similarity sequences of ≥75%, ≥50%, and <50% were considered high, moderate, and low homology, respectively.

2.6. Statistical Analysis

The data were analyzed using the generalized linear mixed models with normally distributed data (Shapiro-Wilk normality test W>0.97575, *P*>0.2959) and an identity link function.

3. Results

PCR products showed clear bands of the 18S rRNA after running on the gel. Accordingly, those products that showed a band were sent for sequencing. DNA sequencing results for each are shown in S1. According to the PCR technique identification and sequencing results, NCBI search showed 42 different fungal species, which were identified in total from the collected birds' feathers. The same species found in more than one bird with *Aspergillus spp*. were the most frequent. Table 1 shows the NCBI matching results for sequencing.

Multiple sequence alignments have been done for the obtained 18S rRNA DNA sequencing results to compare the percentage of similarity among them. Results showed varying percentages of similarities among the identified isolates' 18S rRNA DNA sequence obtained from Barn swallow, White stork, as well as prey and predator after each bird isolate sequence has been aligned together. The results of the percentages of similarities ranged from 39% to 99% (S2). The cladogram clarifies the degree of relatedness among those sequences as illustrated in figures 1-3.

Table 1. Matching results of the sequencing of the PCR products obtained from NCBI blast

Barn swallow	Prey and Predator	White stork
Aphanoascus_fulvescens	*Alternaria spp.*	*Ascomycota spp.*
Arachnomyces_minimus	*Antrodiasinuosa*	*Ascomycota spp.*
Aspergillus flavus	*Ascomycota spp.*	*Aspergillus fumigatus*
Aspergillus fumigatus	*Aspergillus fumigatus*	*Aspergillusniger*
Aspergillusniger	*Aspergillusniger*	*Byssochlamysnivea*
Aspergillusterreus	*Aspergillussustus*	*Chaetomium spp.*
Aspergilluscandidus	*Byssochlamysnivea*	*Monascusfuliginosus*
Auxarthronumbrinum	*Chaetomiumelatum*	*Penicilliumexpansum*
Chaetomium_globosum	*Chaetomiumglobosum*	*Phialosimplexchlamydosporus*
Lichtheimiacorymbifera	*Coniochaetaligniaria*	
Mucorcircinelloides	*Coniochaetavelutina*	
Penicilliumglabrum	*Coprinopsisatramentaria*	
Penicilliumpiceum	*Coriolopsisgallica*	
Thermomyceslanuginosus	*Hyphodermellarosae*	
Trichodermareesei	*Madurellamycetomatis*	
	Monascusfuliginosus	
	Myceliophthorathermophila	
	Myceliophthoraverrucos	
	Penicilliumnamyslowskii	
	Pleosporaceaesp	
	Preussia spp.	
	Psathyrellacandolleana	
	Rhizopusoryzae	
	Schizophyllum spp.	
	Stachybotrysdichroa	
	Thermomyceslanuginosus	

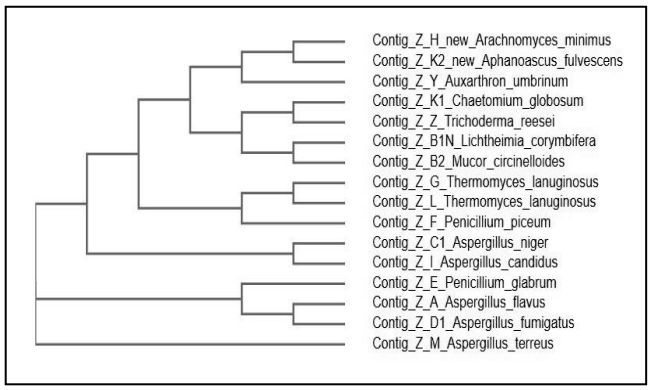

Figure 1. A cladogram design using EMBOSS Clustal Omega showing the most likely evolutionary pathway of 18S rRNA DNA sequencing results of Barn swallow isolates utilizing

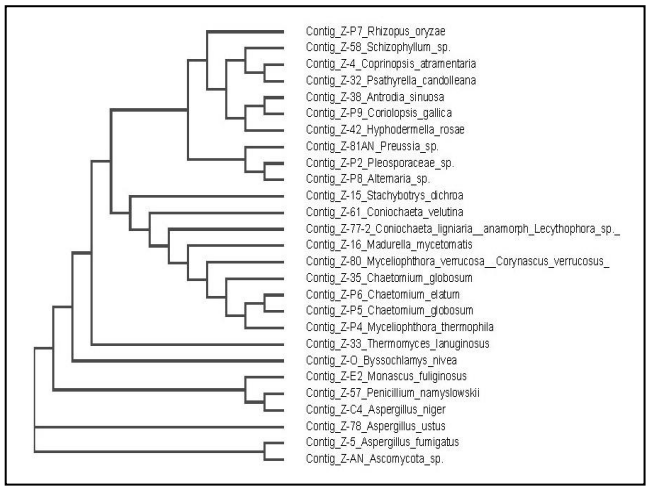

Figure 2. A cladogram design using EMBOSS Clustal Omega showing the most likely evolutionary pathway of 18S rRNA DNA sequencing results of prey and predator isolates utilizing multiple sequence alignments

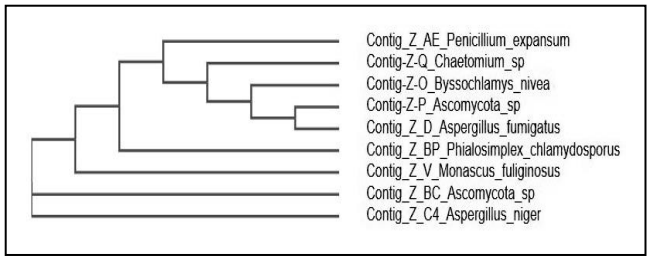

Figure 3. A cladogram design using EMBOSS Clustal Omega showing the most likely evolutionary pathway of 18S rRNA DNA sequencing results of White stork isolates utilizing multiple sequence alignments

As can be observed in figures 1 and 2, the same species showed a high relatedness degree (e.g., *Aspergillus spp., Thermomyces spp.*, and *Chaetomium spp.*). On the other hand, *Aspergillus spp*. showed more relatedness to *Ascomycota spp*. regarding the 18S rRNA sequence, as shown in figure 3.

Moreover, another multiple sequence alignment has been done for the 18S rRNA DNA of the same genus to find out the percentage of similarity among them and whether the host type has an effect on altering the DNA sequence. Results showed that some species have 100% similarity, while other species showed a low percentage of similarity (42%), as shown from figures 4 to 10.

1: Contig_Z_BC_Ascomycota_spWS	100.00	42.49	42.78
2: Contig_Z-AN_Ascomycota_sp.PP	42.49	100.00	99.31
3: Contig-Z-P_Ascomycota_spWS	42.78	99.31	100.00

Figure 4. Percent Identity Matrix created by Clustal Omega showing the percentage similarity of 18S rRNA DNA sequences in *Ascomycota spp*. The 1st shows a low homology, while the 2nd and 3rd show high homology with each species

1: Contig_Z_C4_Aspergillus_nigerWS	100.00	42.96	43.27	43.03	43.02	42.95	43.02	42.92	42.92	42.93
2: Contig_Z_C1_Aspergillus_nigerBS	42.96	100.00	98.24	98.85	99.24	98.54	98.78	98.85	98.85	98.70
3: Contig_Z-78_Aspergillus_ustusPP	43.27	98.24	100.00	99.01	99.01	99.16	99.31	99.31	99.31	99.31
4: Contig_Z_I_Aspergillus_candidusBS	43.03	98.85	99.01	100.00	99.62	99.24	99.46	99.54	99.54	99.54
5: Contig_Z-C4_Aspergillus_nigerPP	43.02	99.24	99.01	99.62	100.00	99.31	99.54	99.62	99.62	99.47
6: Contig_Z_A_Aspergillus_flavusBS	42.95	98.54	99.16	99.24	99.31	100.00	99.62	99.85	99.85	99.54
7: Contig_Z_M_Aspergillus_terreusBS	43.02	98.78	99.31	99.46	99.54	99.62	100.00	99.77	99.77	99.77
8: Contig_Z_D1_Aspergillus_fumigatusBS	42.92	98.85	99.31	99.54	99.62	99.85	99.77	100.00	100.00	99.85
9: Contig_Z_D_Aspergillus_fumigatusWS	42.92	98.85	99.31	99.54	99.62	99.85	99.77	100.00	100.00	99.85
10: Contig_Z-5_Aspergillus_fumigatusPP	42.93	98.70	99.31	99.54	99.47	99.54	99.77	99.85	99.85	100.00

Figure 5. Percent Identity Matrix created by Clustal Omega showing the percentage similarity of 18S rRNA DNA sequences in *Aspergillus spp*. All species show high homology with each other, except for the 1st species which shows a low homology

1: Contig_Z-O_Byssochlamys_nivea-PP	100.00	100.00
2: Contig-Z-O_Byssochlamys_nivea-WS	100.00	100.00

Figure 6. Percent Identity Matrix created by Clustal Omega showing the percentage similarity of 18S rRNA DNA sequences in *Byssochlamys spp*. Both species show a high homology

1: Contig_Z-35_Chaetomium_globosumPP	100.00	98.24
2: Contig-Z-Q_Chaetomium_spWS	98.24	100.00

Figure 7. Percent Identity Matrix created by Clustal Omega showing the percentage similarity of 18S rRNA DNA sequences in *Chaetomium spp*. Both species show a high homology

1: Contig_Z-E2_Monascus_fuliginosusPP	100.00	45.54
2: Contig_Z_V_Monascus_fuliginosusWS	45.54	100.00

Figure 8. Percent Identity Matrix created by Clustal Omega showing the percentage similarity of 18S rRNA DNA sequences in *Monascus spp*. Both species show a low homology

1: Contig_Z_AE_Penicillium_expansumWS	100.00	43.40	43.59	43.37
2: Contig_Z_F_Penicillium_piceumBS	43.40	100.00	97.63	97.48
3: Contig_Z_E_Penicillium_glabrumBS	43.59	97.63	100.00	99.24
4: Contig_Z-57_Penicillium_namyslowskiiPP	43.37	97.48	99.24	100.00

Figure 9. Percent Identity Matrix created by Clustal Omega showing the percentage similarity of 18S rRNA DNA sequences in *Penicillium spp*. All species show high homology with each other, except for the 1st species which shows a low homology

1: Contig_Z_G_Thermomyces_lanuginosusBS	100.00	99.77
2: Contig_Z-33_Thermomyces_lanuginosusPP	99.77	100.00

Figure 10. Percent Identity Matrix created by Clustal Omega showing the percentage similarity of 18S rRNA DNA sequences in *Thermomyces spp*. Both species show a high homology

4. Discussion

This is the first study to track the DNA sequence similarity of 18S rRNA among fungi that infect selected species of birds. When compared to some species, the 18S rRNA sequence has evolved considerably, while remaining conserved in others. Cladogram and percentage of similarity results showed a low homology among the compared species used in this study (<50%) (e.g., *Ascomycota spp, Aspergillus spp, Monascus spp,* and *Ascomycota spp.*) (Figure 3, Figure 4 and Figure 8). A mutation in the 18S rRNA DNA sequence may have caused this evolution, which could be credited to the divergent evolution (14) in case of the same species. On the other hand, the 18S rRNA sequence showed a high percentage of similarity (high homology) among different species within the same genus (>97%), even with other genera. This could be attributed to the convergent evolution (15).

There is no conclusive proof that two or more genes or proteins could be considered homologous, and this remains as presumption. The only way to confirm homology among the genes is through investigating their common ancestor, as well as all intermediate variants. Due to the lack of any fossil evidence of these extinct forms, the basis of the similarity among genes can be taken as an endorsement on homology among them, which is the only observable variable that can be mathematically stated and connected with probability. Therefore, the lower the possibility that two sequences began independently and became accidentally similar, the bigger the similarity ratio between them (8). Accordingly, this study classified the level of homology into high, moderate, and low.

The DNA sequence of the 18S rRNA was used as the marker for the identification of the isolated species used in this study. All eukaryotic cells have 18S rRNA as one of their basic components. Its coding gene is a key marker for random target PCR in environmental biodiversity screening and is one of the most frequently used genes in phylogenetic investigations (16). For this reason, this study utilized the sequencing of the 18S rRNA and use it as a reference for the identification of the related fungal species using the NCBI BLAST tool. One limitation for this technique was that some species could not be identified precisely where it was only mentioned the genus (e.g., *Ascomycota spp*. and *Chaetomiumsp*). Accordingly, it is recommended to conduct further research using biochemical tests to further identify those species.

5. Conclusion

The DNA sequence of the 18S rRNA has been evolved dramatically even within the same species, while still conserved in others. It is a useful tool to be used for the identification of fungal species as it reduces time. Moreover, from the results, there were no comprehensive high homology percentages among the species infecting the same bird.

Authors' Contribution

Study concept and design: M. S. H.

Acquisition of data: M. S. H.
Analysis and interpretation of data: Z. A.
Drafting of the manuscript: S. B.
Critical revision of the manuscript for important intellectual content: M. S. H. and Z. A.
Statistical analysis: S. B.
Administrative, technical, and material support: Z. A.

Ethics

The current study was conducted following the ethical principles of the Mustansiriya University, Baghdad, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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