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Molecular Characterization and Phylogenetic Analyses of Opportunistic Pathogenic Fungi Associated to *Tinea capitis* among pupils from Selected Schools in Somolu Local Government, Lagos state, Nigeria

[1*]SAMUEL, TO; [1] IRECHUKWU, PI; [2]EBABHI, AM; [3]FADIPE, TO

[1]*Department of Botany, Faculty of Science, University of Lagos, Akoka, Lagos State, Nigeria*
[2]*Biology Unit, Distance Learning Institute, University of Lagos, Akoka, Lagos State, Nigeria*
[3]*Department of Biotechnology, Federal Institute of Industrial Research, Oshodi, Lagos State, Nigeria*

**Corresponding Author Email: temmitade@yahoo.com; tosamuel@unilag.edu.ng*
**ORCID: https://orcid.org/0000 0003 2033 5206*
**Tel: +2348036410449*

Co-Authors Email: gini4christ2017@gmail.com; ebabhi.meg@gmail.com; topesalaam@gmail.com

ABSTRACT: *Tinea capitis* is a contagious, scalp-involving dermatophyte infection, with a high prevalence among pre-pubertal children. This present study aims to isolate and characterize (obtaining the sequences of the internal transcribed spacer (ITS) of the genome DNA with the PCR method using universal primers for molecular identification and phylogenetic analyses) of opportunistic mycoses pathogens associated with *Tinea capitis*. Scalp-scrapping were obtained from head lesions of 136 pupils with 85 boys (62.5%) and 51 girls (37.5%) from three primary schools in Somolu local government, Lagos state. One hundred and thirty-three yield fungal growth, of which are twelve fungal species were identified based on their morphological characteristics and nucleotide sequencing of polymerase chain reaction (PCR) using primers targeting the internal transcribed spacer (ITS) regions (ITS1 and ITS4). The identified fungi include: *Aspergillus sydowii*, *Aspergillus niger*, *Canidida orthopsilosis*, *Canidida parapsilosis*, *Chaetomium globosum*, *Neopestalotiopsis* sp and *Talaromyces islandicus*. The utilization of a pairwise nucleotide differentiation, disparity among species was observed, ranging from 394 to 538 base pair (bp). The data reported here is to provide a basis for further improvement on the available mycoses agents (oral/topical) in order to combat opportunistic human pathogenic fungi associated with *Tinea capitis* infection in children. This has become a necessity as this group of fungi pathogens are always present (contribute) at the site of this infection.

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Fungal infections are serious diseases globally (Adesiji *et al.*, 2023). Dermatophytosis is an infection of three genera of fungi, namely *Microsporum*, *Trichophton* and *Epiderphyton* (Buxton *et al.*, 1996). Infections caused by these organisms are referred to as Tinea, this precedes the Latin name for the site they infect. For instance, *Tinea capitis* (ringworm of the scalp), is a scalp-involving dermatophyte infection (Figeuron *et al.*, 1997). *Tinea capitis* can start on the scalp as a non-inflammatory black dot, before the hair shafts around the area start to weaken and hair break off. A well-defined area of hair loss is eventually produced (Bennassar and Grimalt, 2010). *Tinea capitis* is a highly contagious fungi infection, with a high prevalence among pre-pubertal children, and endemicity in several parts of Nigeria (Oladele and Denning, 2014; Afolabi *et al.*, 2018). The problem of *Tinea capitis* fungal disease has become more serious

**Corresponding Author Email: temmitade@yahoo.com; tosamuel@unilag.edu.ng*
**ORCID: https://orcid.org/0000 0003 2033 5206*
**Tel: +2348036410449*

in the last decades, especially in school pupils (Oladele and Denninings, 2014). *Tinea capitis* is widespread in children aged between two and eleven years (Ayaya *et al.,* 2001). This has been attributed to inadequacies in social, economic, healthcare and hygiene practices which include poor living conditions, children's interaction patterns, poor sanitation, housing congestion, limited water supply, and poor health-seeking behavior (VishnuSharma *et al.,* 2015). There is also a higher susceptibility among children who have pets, wet skin conditions, skin injuries, involve in outdoor games, those who walk barefooted, and those who share hairbrushes or unwashed clothing with other people (Ndako *et al.,* 2012). The treatment for this contagious fungal infection may last several weeks or may be difficult to combat (Hay, 2017). Several studies have highlighted the prevalence and causal agents of *Tinea capitis* in several parts of Nigeria (Adesiji *et al.,* 2019), yet there's dearth of information on the opportunistic mycoses pathogens which are always present at the sites of infections. These cosmopolitan opportunistic fungi have very low inherent virulence thereby cannot initiate an infection but are always present at already established infectious sites (Walsh *et al.* 1995). It is thereby necessary to isolate and identify these group of fungi as they are very important in the achievement of effective treatment of this type of mycoses. This present study therefore aimed at obtaining the sequences of the internal transcribed spacer (ITS) of the genome DNA with the PCR method using universal primers for molecular identification and phylogenetic analyses of these group of fungi. These sequences will then be deposit at GenBank (NCBI) for public access.

MATERIALS AND METHODS

Study area and population: The study was carried out in three selected public schools in Bariga among pre-adolescent children of ages 6-11 years old. Bariga is a district and suburb in Shomolu Local Government Area of Lagos state, southwestern Nigeria. It is a geographical defined within latitudes 6°31′20″ and 6°33′30″ North and longitudes 3°22′0″ and 3°24′0″ East. For ethical consideration, permission was sought from and granted by the school administration and participants' guardians/parents. The pupils in each school were screened for clinical signs of *Tinea capitis*. Signs like scaling, lesions, and hair loss. A total of 574 pupils from the three schools were screened, of which specimen was collected from only 136 pupils with clinical signs of *Tinea capitis.*

Collection of specimen/ Fungal Isolation: The affected head area was cleaned with 70% ethanol and samples were obtained by scraping the affected area with a sterile toothed comb, one for each child (Plate 1-7). The scrapings were collected separately into sterile papers, folded on edges to form flat packet and these were labelled with pupils' data. The collected samples were transported to the Mycology Unit, Department of Botany, University of Lagos for culturing/processing. With the aid of tweezers, some pieces of the collected scrap were inoculated onto the surface freshly prepared Sabourauds dextrose agar (SDA) plates with chloramphenicol (no addition of cycloheximde). These were incubated at room temperature (30 ℃) which was observed daily for fungal growth. This process was repeated until pure plates of isolates were obtained.

Plate 1-7: Pupil's head with different *Tinea capitis* symptoms

Extraction/spectrophotometric/gel electrophoresis analysis of DNA: The isolated fungi were aseptically transferred from stock-culture into tubes containing Sabourauds dextrose broth (SDB) and incubated at room temperature for five days.

The spore and mycelium of pure cultures were harvested for DNA extraction. The DNA extraction procedure of Zymo Research Bacterial/Fungi kit by Inqaba Biotec (South Africa) was adopted.

The DNA samples were slightly vortexed and spun down using a Cleaver Scientific Mini micro-centrifuge and 1µl was used to determine the purity and concentration against the elution buffer as blank. The ratio of absorbance at 260 nm and 280 nm and concentration was recorded. The quality of extracted genomic DNA was assessed in a 1 % (w/v) ethidium bromide-stained agarose gel. A 1 kb DNA ladder (Solis BioDyne) was run as the molecular weight marker. The electrodes were connected to the power

pack in such a way that the cathode was positioned by the well. The run parameters were 90 volts for 1 hr. The gel was documented in a Cleaver Scientific OMNIDOC System.

Polymerase Chain Reaction (PCR): Amplification of the ribosomal subunits was performed according to the New England Biolabs ITS1. Using One Taq quick load 2x master mix with standard buffer and the primers ITS1 (5'TCC GTA GGT GAA CCT TGC GG 3) and ITS4 (5'TCC TCC GCT TAT TGA TAT GC 3'). The PCR reaction mixture (30 µl) containing 15 µl PCR master mix (New England Biolabs), 1 µl (10µM) of each primer, 7.5 µl nuclease free water and 4.5 µl template DNA 1.5 µl (10nm) of (ITS1 and ITS4). PCR amplification was performed in cleaver scientific G-TC 965 following conditions 94 ℃ for 30 seconds; 94 ℃ for 30 seconds; 52 º C for 1 minute; 68 º C for 1 minute; 68 º C for 5 minutes; 4 º C hold in Cleaver Scientific GTC 96 S. PCR products were separated on 2% agarose gel, stained with ethidium bromide.

Sequencing and phylogenetic analysis: The PCR amplicons of all fungal isolates were sequenced in Inqaba Biotec (South Africa). The sequences were trimmed and edited using Sequencher 5.4.6. Build 46289. Sequence Homology was done using the NCBI-BLAST tool and alignment using ClustalW in MEGA 7. Phylogenetic analysis was done using the Tamura-Nei and p-distance algorithms of the Neighbour-Joining method considering 1000 bootstrap replications in MEGA 7. The sequences have been submitted to the NCBI-GenBank and accession numbers issued.

RESULTS AND DISCUSSION

Of the 136 collected samples from the participants, three samples did not yield any visible fungal growth on culture plates despite being obtained from lesion area of *Tinea capitis.* The 136 participants consisted of (85) 62.5 % boys and (51) 37.5 % girls while a yield of 133 isolates were morphologically observed, and found to belong to twelve different fungal species. Extracted genomic DNA from the twelve fungal isolates showed intact bands which indicated that the extracted DNA is suitable for further downstream analysis (Plate 8). The purity of the extracted DNA was within the range of 1.8 - 2.0 ng/ul as expected for pure DNA and the agarose gels of PCR amplicons showed products ranging from 500 base pairs to 600 base pairs (Plate 9) as expected for a successful amplification of the internally transcribed spacer regions of fungal species.

After a preliminary identification, the ITS sequences of the extracted fungi obtained were analyzed to find their identical. The sequences revealed that six out of the twelve isolates (SIF 1, 2, 6, 7, 8 & SIF 9), belong to the genus *Aspergillus*. Others were identified as genera *Trichoderma* (SIF3), *Neopestalotiopsis* (SIF4), *Chaetomium* (SIF5), *Taloromyces* (SIF10) and *Candida* (SIF11 and SIF12). SIF 1, 6, 7, 8 & SIF 9, all have closet homology with *Aspergillus sydowii*. Although, these isolates differ morphologically (culture plates), while SIF2 was found to be homology with A. *niger* (109INT-1.4.4). Multiple sequence alignment analysis of isolates SIF1 & SIF8 revealed these two to be similar with reference AsN19C03 (China) at the GenBank data (Table 1). SIF 6, 7 & 9 were aligned to be similar with reference DTO254H7 (South Africa) at the GenBank database (Table 1)..

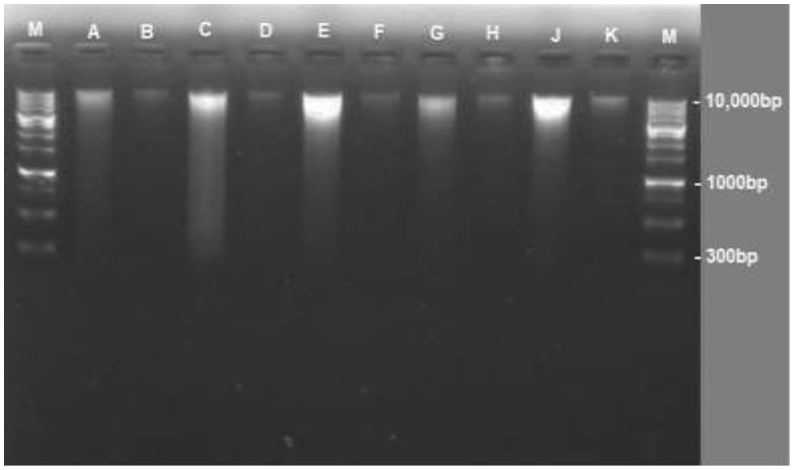

Plate 8: Gel electrophoresis of DNA Extracted from Fungal Samples
Lane M= 1kb DNA Ladder, Lane A to K = DNA band of Fungal isolates

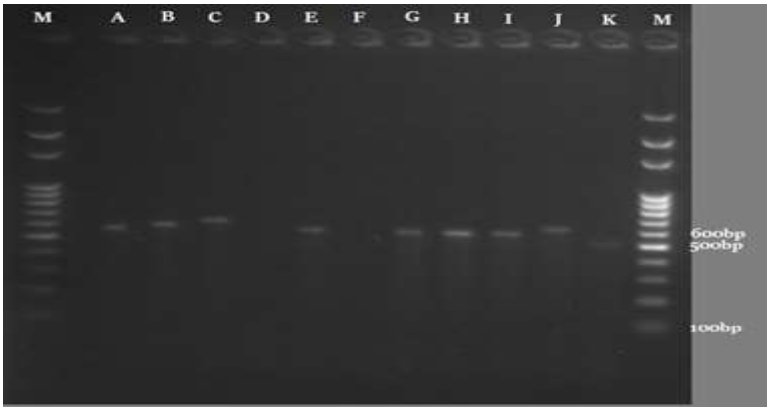

Plate 9: PCR Amplicons Agarose Gel
M = 100bp DNA Ladder, Lane 2 to Lane 12 = Amplicons from Samples AP - MP

Table 1: Locations of Strains with Closet Homology from NCBI-BLAST and NCBI-GenBank Submission Information

S/N	Isolate Code	Organism with Closet Homology	Identity	Location Isolated	Nucleotide sequences (bp)	Accession Number
1	SIF1	*Aspergillus sydowii* AsN19C03	100 %	China	508	MN700115
2	SIF2	*Aspergillus niger* 109INT-1.4.4	99.81 %	Vietnam	538	MN700116
3	SIF3	*Trichoderma longibrachiatum* NCQ3-2	100 %	China	533	MN700117
4	SIF4	*Neopestalotiopsis* sp FX24	99.34 %	China	495	MN700118
5	SIF5	*Chaetomium globosum* CES5	100 %	India	431	MN700119
6	SIF6	*Aspergillus sydowii* DTO245H7	100 %	South Africa	513	MN700120
7	SIF7	*Aspergillus sydowii* DTO245H7	99.81 %	South Africa	516	MN700121
8	SIF8	*Aspergillus sydowii*	100 %	China	457	MN700122
9	SIF9	*Aspergillus sydowii* DTO245H7	100 %	South Africa	505	MN700123
10	SIF10	*Talaromyces islandicus* NWUSeq40	100 %	South Africa	438	MN700124
11	SIF11	*Canidida parapsilosis* CMC_182	99.75 %	Italy	394	MN700125
12	SIF12	*Canidida orthopsilosis* Milk3	100 %	Brazil	429	MN700126

Isolates SIF11 and SIF12 showed closet homology with *Canidida parapsilosis* (CMC_182, Italy) and *Canidida orthopsilosis* (Milk3, Brazil) respectively. Isolates SIF3, SIF4 and SIF5 are homology similar with *Trichoderma longibrachiatum* (NCQ3-2, China) Table 1), *Neopestalotiopsis* sp (FX24, China) and *Chaetomium globosum* (CES5, India) respectively. Isolate SIF10 showed 100 % homology with *Talaromyces islandicus* (NWUSeq40, South Africa (Table 1). The accession numbers of the twelve fungal isolates issued by the NCBI-GenBank, the sizes of submitted sequences and their new NCBI-GenBank codes are summarized in table 1.

Tinea capitis is a contagious disease of the scalp that is common among children and it is prevalent in this part of the globe (Nigeria). Associated with this fungal infection are some opportunistic non-dermatophytes. It is therefore necessary to identify these non-dermatophytes for effective control and treatment of this disease. The results of this study, showed no growth of dermatophytes in the samples collected, which may be due to the lack of addition of cycloheximide (an antibiotic used to inhibit the growth of opportunistic moulds that hamper the recovery of dermatophytes) to the growth medium used for isolation. Plus, the fact that the fungi of interest (opportunistic mycoses) are fast growing group of fungi which grow within five to seven days of inoculation on growth medium thereby exhausting the nutrient, leaving no space to other group of fungi to be recovered. These non-dermatophytes (particularly *Aspergillus*) have been reported by Maruthi *et al.* (2007) who stated in their report that the presence of other non-dermatophytes may be due to the ubiquitous nature of their spores in our environment. These authors, also stated that these fungi were recovered due to the contact of the school pupils with playground soil. Most of the identified fungi in this present work have been reported to have affinity for soil (Pandey *et al.* 1990). Ndako *et al.* (2012) in their report stated that, the confirmation of both dermatophytic and non-dermatophytic fungal isolates from Tinea infected sampled population suggests a relationship that requires further investigation. They affirmed that the complete understanding of this relationship will have a strong implication on control/treatment of dermatophytosis in children while Maruthi *et al.* (2007) emphasized that the presence of these opportunistic mycoses should be considered

significant when considering effective approaches to the treatment of Tinea. From the sampled population, 62.5 % were male compared to 37.5 % of female. This could probably be due to the fact that male children are more prone to have contact with sand by engaging in outdoor activities. The results of Ndako *et al.* (2012) supports this result by stating that physical engagement of male children in contact sports such as wrestling, football, boxing and tag games are factors that pre-dispose males to high prevalence of superficial fungal infections. Also, Adou-Bryn *et al*. (2004) collaborated this study by stating that the low prevalence rates in girls could be associated with the facts that girls weave their hair and have high general personal hygiene. However, this contradicts Dogo *et al.* (2020) who reported that the prevalence of *Tinea capitis* among girls was higher (51.4%) than that among boys (41.5%) but not significantly different. In this study, it was observed that the *Aspergillus* isolates which were morphologically different on culture plates are same species after being subjected to NCBI-BLAST. An indication that morphological features of the fungal isolates were only useful for the identification of these isolates to the genus level. This is in consonance with the findings of Lutzon *et al.* (2004), who stated that morphological features are not adequate to provide fungal identities to species level which is very important as it is one of the crucial steps to aid the clinician in initiating prompt and appropriate antifungal agent.

The ITS sequences of the isolated fungi helped in identifying the isolates to the species level. This probably can assist in proper diagnosis and treatment of the disease. The reports of Graser *et al.* (1999) and Hirai *et al.* (2003), also support the use of the ITS region as a focus for phylogenetic analysis and identification of species molecularly which they noted has not only contributed to a revision of the taxonomy of fungi, but also provided deeper understanding of the evolution and insights into the identification, taxonomy, and epidemiology of the species/strains. *Aspergillus sydowii* which was noted to have differed morphological features (SIF 1, 6, 7, 8 and SIF 9) was shown through the ITS sequencing to belong to a single species. This organism is predominantly a saprophytic fungus found in soil but have been implicated in several human diseases like aspergillosis and keratomycosis (Hoog, 2000). One of the opportunistic mycosis pathogens identified in the present study is *Canidida parapsilosis.* Trofa *et al.* (2008) had reported this species as one of the normal human commensals which is usually isolated from the subungal space of human hands and reported by Guo *et al.* (2021) as one of the causal agents of invasive candidiasis. The identity of *Trichoderma longibrachiatum* was also confirmed in this study. *T. longibrachiatum* is found globally in the soil particularly in warm climates and have been linked to skin and skin structural infection, keratitis, and septic shock by Zhang and Li, (2019). *Chaetomium globosum* which was also identified in this present study had previously been implicated as an opportunistic pathogenic fungus by Guarro *et al.* (1995). These authors studied the pathogenicity and antifungal susceptibility of Chaetomium species and reported that *C. globosum* was the most common species isolated in about nine clinical cases of infection while Kim *et al.* (2013) reported this species as a human allergens and opportunistic agents of ungual mycosis and neurological infections. *Talaromyces islandercus* which was also isolated had not been clinically linked to infections although it is a soil inhabiting species unlike other species like *Talaromyces marneffei* which had been clinically linked with talaromycosis infections. For effective and appropriate treatment and control of dermatomycosis there is need for accurate and adequate identification of causal and enhancing agents which can be achieved through molecular laboratory tools that provide a rapid and accurate method for identification of fungal species.

Conclusion: In the present study, the accurate identities of opportunistic human pathogenic fungi associated with Tinea infection were assessed based on molecular identification methods. The data reported here is to provide an update on the existing information on this group of fungi/pathogen in order to combat *Tinea capitis* infection in children. This has become a necessity as this group of fungi pathogens are always present (contribute) at the site of this infection.

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