

MOLECULAR TYPING OF DERMATOPHYTES ISOLATED FROM PUPILS AND STAFF MEMBERS OF SOME SELECTED SCHOOLS IN ANAMBRA STATE, NIGERIA

Abstract

Dermatophytes are the fungal pathogens of human and animals infecting the keratinized tissues of the body namely skin, hair and nails. They include species of *Trichophyton*, *Microsporum* and *Epidermophyton*. This study was aimed at molecular typing of dermatophytes isolated from school pupils and staff members of some selected Schools in Anambra East and Ayamelum L.G.A^s of Anambra State in Nigeria. One thousand(1000) samples (scalp/hair, nail, feet, glabrous skin and groin/perianal) were collected from pupils and staff members of both gender and ages of 1 to 60 years that showed visible signs of skin infection located in these two Local Government Areas. Molecular studies were used as confirmatory tests on dermatophytic isolates using Sanger sequencing method. The results show that the dermatophytic isolates includes: *Trichophyton tonsurans*, *Microsporum audouinii*, *Trichophyton rubrum*, *Microsporum canis*, *Trichophyton violaceum*, *Trichophyton verrucosum*, *Trichophyton mentagrophytes*, and *Epidermophyton floccosum*. Results also revealed the nucleotide sequences of the dermatophytes and genetic relationships between isolated dermatophytes from different pupils and schools. PCR-RFLP maybe considered as gold standard for the diagnosis and Confirmation of Source of infection of dermatophytes and can aid in initiating prompt and appropriate antifungal therapy.

Keywords: Antifungal Sequencing, Dermatophytes, Molecular typing, Anambra State.

1. INTRODUCTION

Dermatophytes are the fungal pathogens of humans and animals infecting the keratinized tissues involvement like skin, nails and hairs (1). Dermatophytoses are fungal infections that specially only superficial keratinized tissues of the body, skin, hair, nails (2). Dermatophyte infection is generally cutaneous and restricted to the non-living cornified layers due to of the unable of the fungi to penetrate the deeper tissues or organs of immunocompetent hosts (3). These cutaneous mycoses are the most common fungal infection of man and are often called Tinea or ringworm (4).

Dermatophytes are transmitted mostly by direct contact with infected host (human or animal) or by direct or indirect contact with infected exfoliated skin or hair in clothing, combs, hair brushes, theatre seats, caps, furniture, bed linens, shoes, socks, towels, hotel rugs, bath house, and locker room floors (1). Depending on the species, the organism may be active in the environment for up to 15 months. These fungi have worldwide distribution and at present there are 40 recognized species in the dermatophyte genera. About 25 species belonging to the genera *Epidermophyton*, *Microsporum* and *Trichophyton* are presently known to infect man (5). But recently up to 40 species are found to infect man (6).

The problem of accurate definition and characterization of microorganisms (bacteria, fungi, viruses, parasites,) has always been of great important in phylogenetic analysis and taxonomic identity, clinical microbiology and epidemiology. Initially, the study of dermatophytes was based on colony and microscopic morphology, but identification of these keratinophilic organisms was sometimes difficult because of their overlapping characteristics, variability and pleomorphism.

Phylogenetic relationships and species-specific sequences could not be entirely defined by the previous methods, more technique known to resolve genetic disjunctions between very closely related species was used. Sequencing of the hyper variable internal transcribed spacer (ITS) region, consisting in Ascomycetes of ITS 1, ITS 2 and the intermediary 5-8S rDNA, was a choice tool allowing the elucidation of the phylogeny of closely interrelated filamentous fungi (7). A total of 54 strains belonging to 41 recognized species and varieties of the family *Arthrodermataceae* have been studied using this procedure (8). The studies showed that geophilic species were highly separated from the other members of the *Arthrodermataceae*. In contrast, the genera *Trichophyton* and *Microsporum* were not resolved in the phylogeny obtained with this marker. The geophilic species of *Stockdaleae* was shown to be in need of reclassification. *M. canis*, *M. Audouinii* and *M. equinum* were found to be very closely related. The *T. mentagrophytes* complex and its *Arthroderma*-related teleomorphs were classified into three groups: *T. mentagrophytes*, a Neotypified *T. interdigitale* related to *A. Vanbreu seghemii* and *T. erinacei* related to *A. benhamiae*. To accurately investigate the taxonomic structure of the *T. mentagrophytes* complex and *T. tonsurans*, the same authors studied 24 related species or varieties using ITS sequencing (9). Their analysis revealed that these 24 taxa, many of them long-disused older names, could be reduced to only five species, including the three pre-defined members of the *T. mentagrophytes* complex, as well as *T. simii*, the anamorph of *A. simii*, and *T. tonsurans*. These data are in agreement with genotypic results obtained by mtDNA restriction fragment length polymorphism (RFLP) (10), *CHS1* gene sequencing (11) and ITS 1 sequencing (10), in line with previous phenotypic observations. In addition,(1) found that physiological criteria are not in conflict with genetic groupings for most of the strains studied.

Laboratory diagnosis of dermatophytosis requires the association of a positive direct examination of clinical specimens showing characteristic septate hyphae and the dermatophyte cultural isolation on Sabouraud medium. Since direct examination does not allow species identification and dermatophytes

generally grow in 1–3 weeks, new techniques allowing a fast and reliable diagnosis of dermatophytosis combined with species identification could be valuable. In addition, several keratinophilic organisms classically responsible for superficial and mild infections had been reported from deep-seated infections in patients with severe immunodeficiency (12).

The gene coding for the small ribosomal subunit (18SrRNA) is highly conserved and is thus a choice tool for detection of fungi, followed by hybridization with species- specific oligonucleotides. This PCR-based methodology was used by (12) in 69 skin and nail specimens allowing diagnostic distinction between dermatophyte and *Candida* infections. Restriction analysis of the PCR products could distinguish between dermatophytes, yeasts and moulds. The sensitivity of the technique, calculated at 92%, was higher than that of culture (73%). However the use of these techniques in clinical material is of debatable value. Skin, hair and nail are not sterile sites and, for example, arthroconidia could possibly be detected in the scalps of uninfected subjects close to a child with *Tinea capitis*. In these cases, PCR would not be able to distinguish contamination from infection.

Therefore, the purpose of the present work was to undertake molecular typing of dermatophytes isolated from pupils and staff members of selected schools in Anambra East and Ayamelum L.G.As of Anambra State, Nigeria.

2. MATERIALS AND METHODS

2.1 Study Population

The study design was convenient sampling method. There are 21 Local Government Areas in Anambra State of Nigeria. Ayamelum and Anambra East Local Government Areas are included and are situated in lowland swampy area of the state. Seventeen schools were randomly selected in the two Local Government Areas sampled population were derived.

This study was conducted in Nursery and Primary Schools in Ayamelum and Anambra East Local Government Areas. Samples were collected from school children, adolescents and some staff that are adults.(School A-Q). Five hundred (500) samples each were collected from males and females of ages from 1year to 60 years. Ayamelum and Anambra East L.G.A has moderate population with most of the inhabitants being traders, farmers, fisherman, civil servants and artisans but predominately farmers. “Omanbala River” which also is called Anambra River encloses Anam, Umueri, Aguleri and Nando and flows into the River Niger and this made it possible for about 10% of them being fishermen.

2.2 Culture of Sample

Clinical samples of hair, skin, nail and groin region collected from participants were subjected to molecular typing method.

2.2.1 Isolation: Sterile petri plates containing dermatophyte test medium were inoculated with scalp scraping and hair stubs collected from infected pupils. This medium is a selective and chromogenic medium that permits the growth of dermatophytes and these organisms give the medium a reddish coloration. These plates were incubated at room temperature of between 25 – 27°C for up to 10 days during which the plates were observed for growth. Each fungal growth was subcultured on SDA to obtain a pure culture which was then stored in agar slants for further studies.

2.2.2 Identification: Colonial morphology on dermatophyte test medium was used for preliminary identification of the dermatophytes. Pure fungal colonies were also subjected to lactophenol blue staining for microscopic observation of specialized hyphae and the morphology of their macronidia, micro conidia and chlamydo spores.

2.3 Molecular typing of dermatophytic isolates

2.3.1 DNA Extraction

Genomic DNA was extracted from the dermatophytic clinical samples using Quick-DNA™ Fungal Miniprep Kit (Zymo Research), according to recommended protocol. Fungal cells (50 mg/wet weight) were re-suspended in up to 200 µl of isotonic buffer (PSB) to a ZR Bashing Bead Lysis Tube (0.1mm & 0.5mm). Then 750 µl Bashing Bead™ Buffer was added to the tube. This was secured in a bead beater fitted with a 2 ml tube holder assembly and processed at maximum speed for ≥ 5minutes. The ZR Bashing Bead™ Lysis Tube (0.1 & 0.5mm) was centrifuged in a microcentrifuge at 10,000 x g for 1 minute. Up to 400 supernatant was transferred to a Zymo-Spin™ III-F Filter in a Collection Tube and centrifuged at 8000 x g for 1 minute. A 1,200 µl of Genomic Lysis Buffer was added to the filtrate in the Collection Tube. And 800 µl of the mixture was transferred to a Zymo-Spin™ IIC Column in a Collection Tube and Centrifuged at 10,000 x g for 1 minute. The flow through the Collection Tube was discarded. A 200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC Column in a new Collection Tube. A 500 µl g-DNA Wash Buffer was added to the Zymo-Spin™ IIC Column and centrifuged at 10,000 x g for 1 minute. The Zymo-Spin™ IIC Column was transferred to a clean 1.5 ml microcentrifuge tube and 100 (35 minimum) DNA Elution Buffer added directly to the column matrix, and centrifuged at 10,000 x g for 30 seconds to elute the DNA.

2.3.2 PCR Protocol

Ten(10) µl of One *Taq* Quick-Load 2X Master Mix with Standard Buffer (New England Biolabs Inc.); 1 µl each of forward and reverse primers; 7 µl of Nuclease free water and 1µl of DNA template was used to prepare 20 µl reaction volume of the PCR cocktail.

The reaction was gently mixed and transferred to a preheated thermal cycler. Amplification conditions for the PCR were as follows: 5 min at 94°C to denature the DNA, followed by 35 cycles of denaturation

at 94⁰C for 30 secs, primer annealing at 50⁰C for 30 secs and strand extension at 68⁰C for 10 minutes on an Eppendorf nexus gradient Mastercycler (Germany). PCR products were separated on a 2% agarose gel and DNA bands were visualized with syber gold.

2.3.3 Sequencing Protocol

PCR products were cleaned using ExoSAP Protocol as follows:

The Exo/SAP master mix was prepared by adding the following to a 0.6ml micro-centrifuge tube:- 50µl Exonuclease I (Catalogue No. NEB M0293L) 20 U/ul; and 200µl Shrimp Alkaline Phosphatase (Catalogue No. NEB M0371) 1 U/ul

The following reaction mixture was prepared:

10µl Amplified PCR Product and 2.5µl of ExoSAP Mix (step 1)

This was mixed well and was incubated at 37⁰C for 15 min

The reaction was stopped by heating the mixture at 80⁰C for 15 min

Fragments were sequenced using the Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000 according to manufacturer's instructions:

<https://www.nimagen.com/products/Sequencing/Capillary-Electrophoresis/BrilliantDye->

Terminator-Cycle-Sequencing-Kit/

The labelled products are then cleaned with the ZR-96 DNA Sequencing Clean-up Kit (Catalogue No. D4053):

<http://www.zymoresearch.com/downloads/dl/file/id/52/d4052i.pdf>

The cleaned products were injected on the Applied Biosystems ABI 3500XL Genetic Analyser with a 50cm array, using POP7

<https://www.thermofisher.com/order/catalog/product/4406016>

Sequence chromatogram analysis was performed using FinchTV analysis software:

<https://www.softpedia.com/get/Science-CAD/FinchTV.shtml>

3.RESULTS

Nucleotide sequences of *Trichophyton tonsurans* isolate from scalp lesions isolated from 26 year – old

male members of staff in school H; from 8 year – old female pupil in school E; from groin lesion of a 53 year – old staff female in school L and nucleotide sequences of *Microsporum audounii* isolate from scalp lesion of a 14 year – old male pupil in school K; and from skin lesion of a 44 year – old male, pupil in school B and nucleotide sequences of *Trichophyton rubrum* isolate from scalp lesion of a 12 year – old female pupil in school A and from nail lesion of a 39 year – old male staff at school N and nucleotide sequences of *Microsporum canis* isolated from skin lesion of a 7 year – old female pupil attending school H and nucleotide sequences of *Trichophyton violaceum* isolated from scalp lesion of a 22 year – old male staff working in school A and nucleotide sequences of *Trichophyton verrucosum* isolated from scalp lesion of a 6 year – old male pupil in school O is indicated and the nucleotide sequences of *Trichophyton mentagrophytes* isolated from scalp lesion of a 13 year – old female pupil attending school K and nucleotide sequences of *Epidermophyton floccosum* isolated from skin lesion of a 7 year – old pupil in school B are indicated.

Phylogenetic Tree

The phylogenetic tree portrays the genetic relatedness of the dermatophytic isolates. This is achieved by using paralogues and homologues. The paralogues showed that the organisms are not related while homologues showed that the isolates have common ancestors: Dermatophyte isolate 13 (*Epidermophyton floccosum*) is paralogue to dermatophytic isolates 5 (*Microsporum audounii*); Dermatophyte isolate 2 (*Microsporum audounii*) and Dermatophyte isolate 10 (*Microsporum canis*).

Dermatophyte isolate 15 (*Trichophyton rubrum*) has homologues relationship with dermatophytic isolates 35 (*Trichophyton mentagrophytes*), 21 (*Trichophyton violaceum*), 14 (*Trichophyton verrucosum*), 6 (*Trichophyton tonsurans*) and (*Trichophyton rubrum*). They are all paralogue of dermatophytic isolates 1 (*Trichophyton tonsurans*). Hot map revealed the individual genetic relatedness of the dermatophytic isolates from this study (figures 1 to 10).

NB: The phylogenetic tree and the hot map aided in the analysis of the genetic relatedness of the source of the dermatophyte isolates from the area studied.

Trichophyton tonsurans CBS 645.80 ITS region; from TYPE material
 Sample ID: NR_147423.1

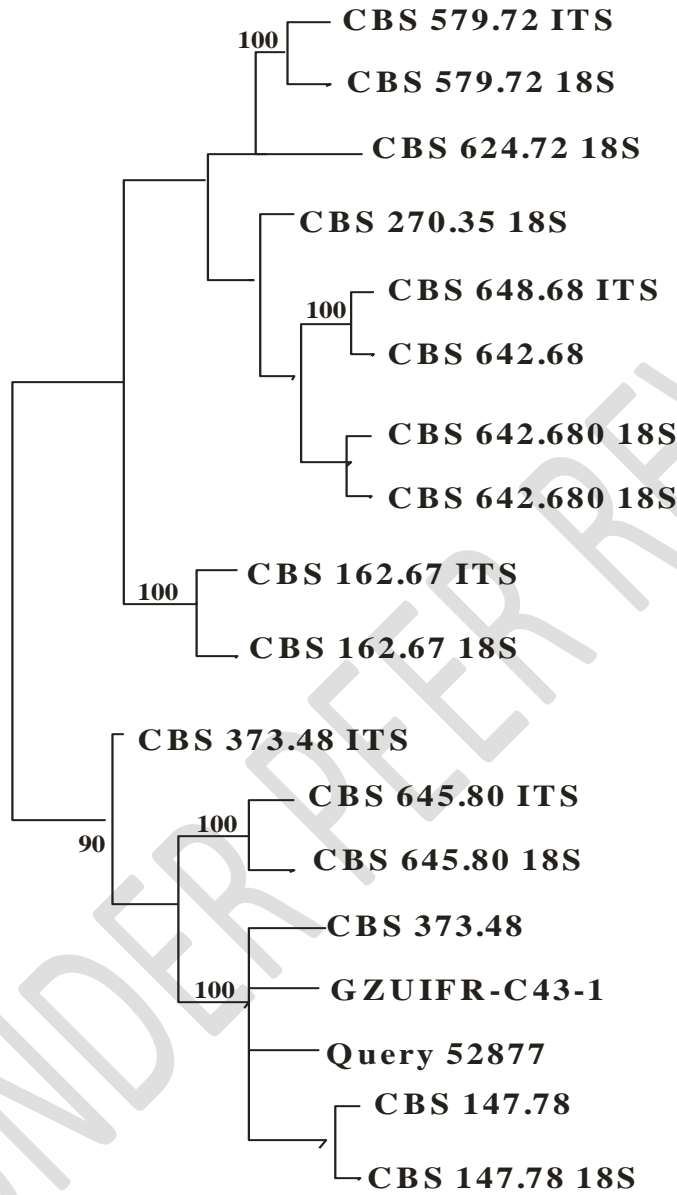


Figure 1: Phylogenetic tree of *Trichophyton tonsurans* isolated from scalp lesion of a 26 year – old male staff working in school H.

Trichophyton tonsurans CBS 645.80 ITS region; from TYPE material
Sample ID: NR_147423.1

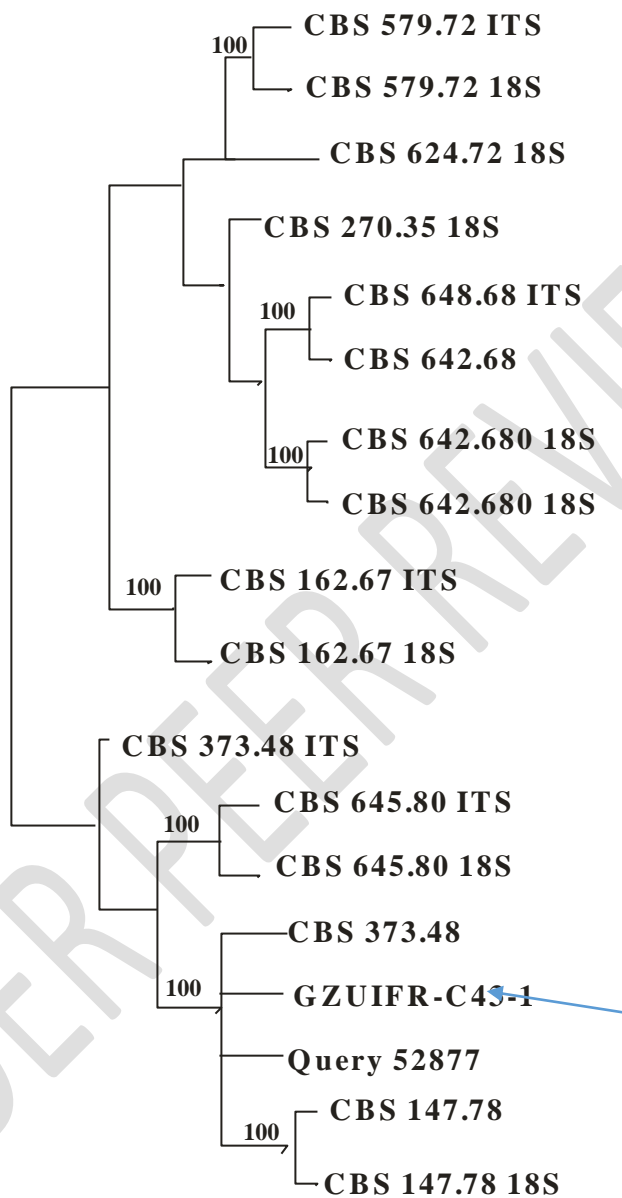


Figure 2: Phylogenetic tree of *Trichophyton tonsurans* isolated from groin lesion of a 53 year – old female staff teaching in school L.

Microsporium audounii NRRL 6161 ITS region; from TYPE material
 Sample ID: NR_135385.1

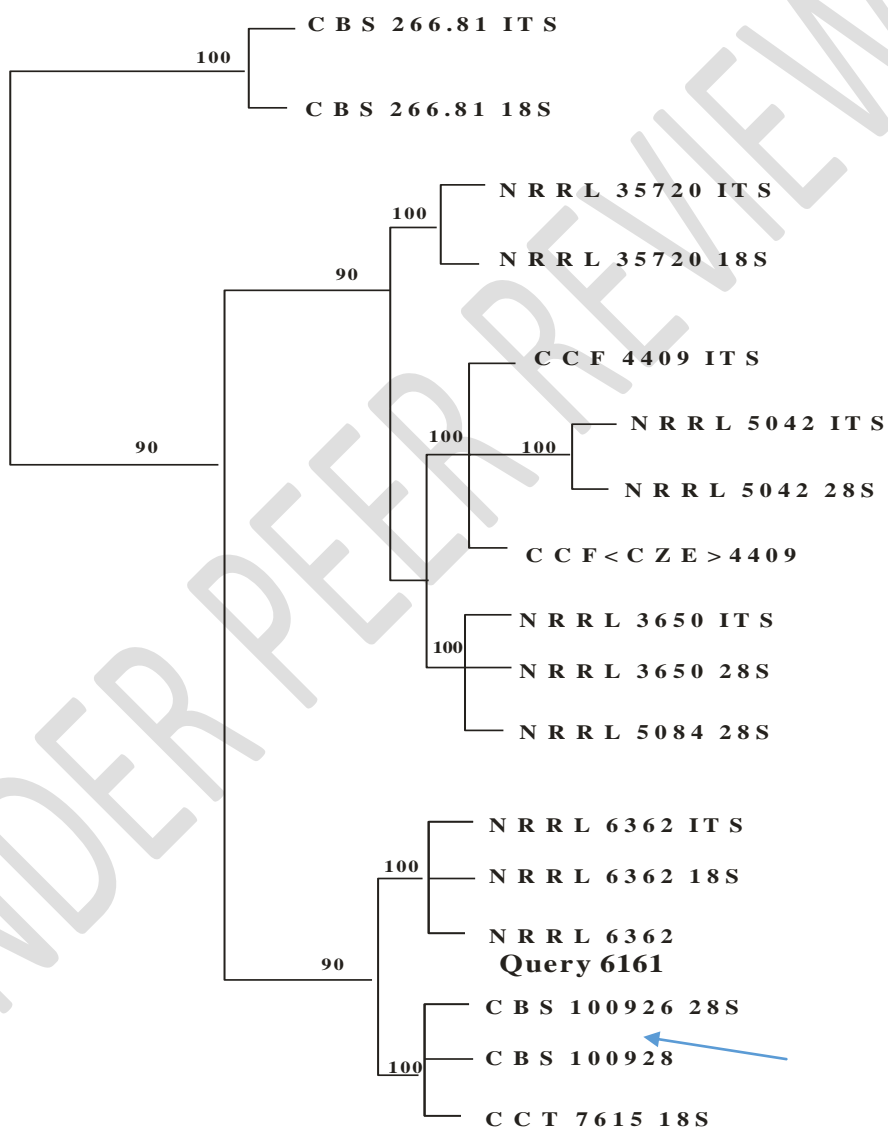


Figure 3: Phylogenetic tree of *Microsporium audounii* isolated from scalp lesion of a 14 year – old male pupil attending school K.

Microsporium audouinii NRRL 6161 ITS region; from TYPE material
 Sample ID: NR_135385.1

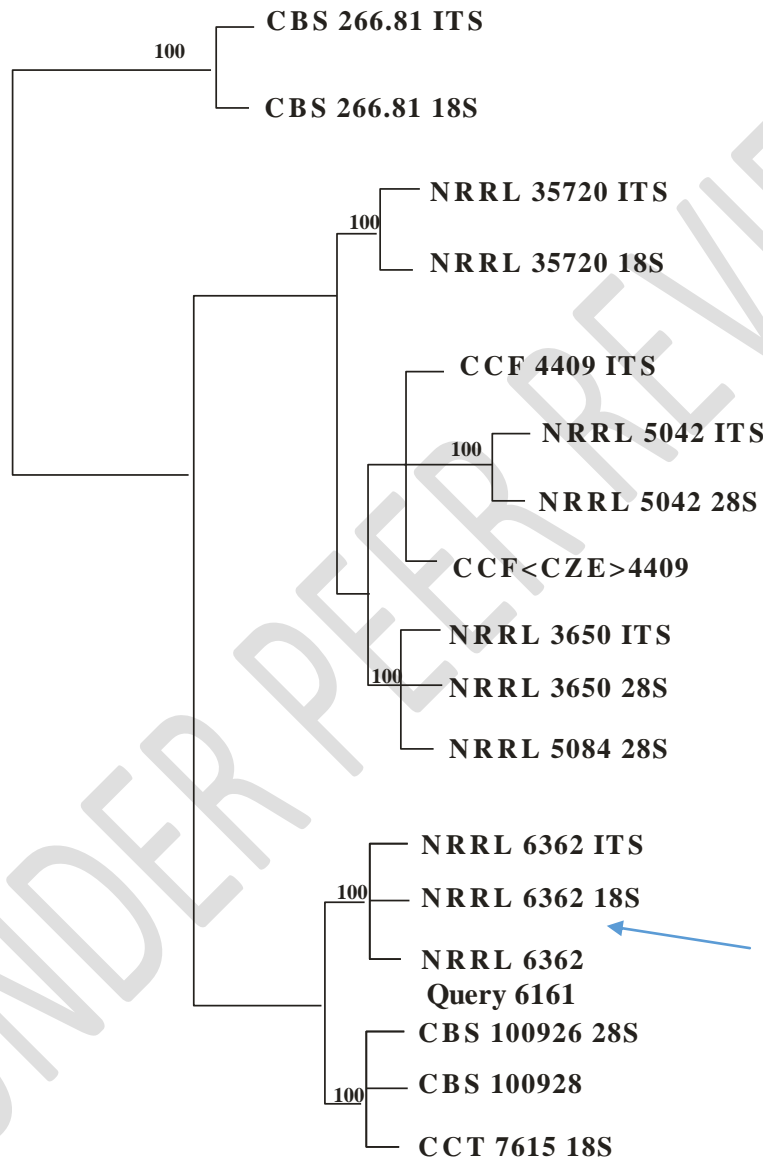


Figure 4: Phylogenetic tree of *Microsporium audouinii* isolated from skin lesion of a 44 year – old male pupil in school B.

Trichophyton rubrum strain GZUIFR - C43 - 1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene complete sequence; and internal transcribed spacer 2, partial sequence.
Sample ID: EU004812.1

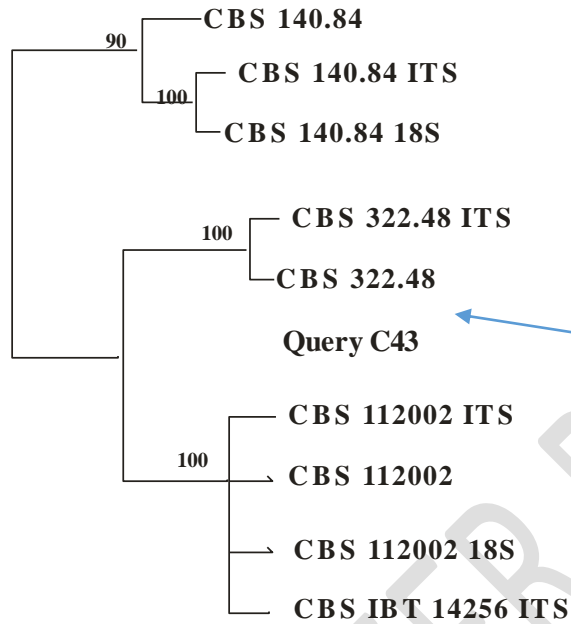


Figure 5: Phylogenetic tree of *Trichophytonrubrum* isolated from scalp lesion of a 12 year – old female pupil attendingschool A.

Trichophyton rubrum strain GZUIFR - C43 - 1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene complete sequence; and internal transcribed spacer 2, partial sequence.
Sample ID: EU004812.1

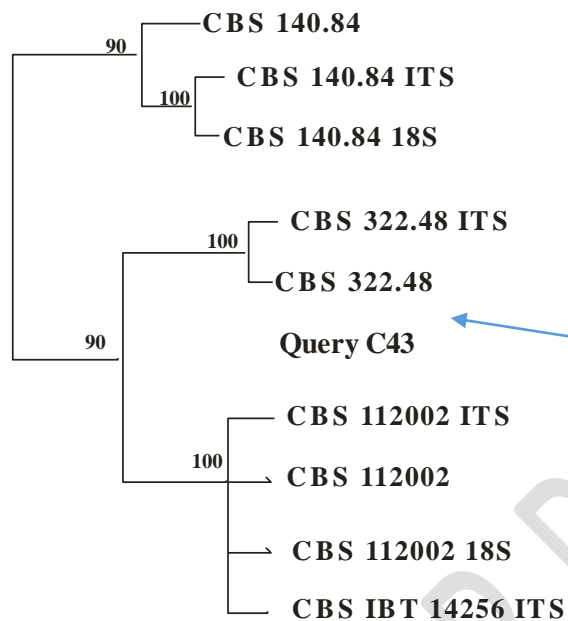


Figure 6: Phylogenetic tree of *Trichophyton rubrum* isolated from nail lesion of a 39 year – old male staff working inschool N.

Microsporium canis NRRL 6161 ITS region; from TYPE material
Sample ID: NR_135385.1

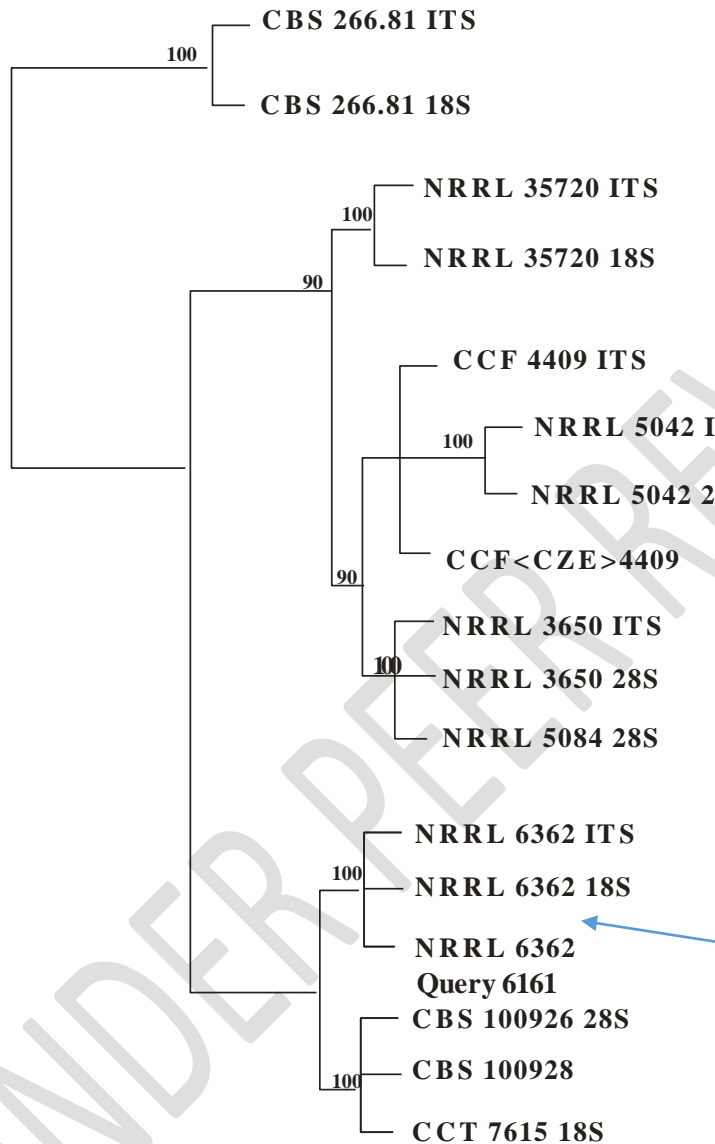


Figure 7: Phylogenetic tree of *Microsporium canis* isolated from skin lesion of a 7 year – old female pupil in school H.

Trichophyton violaceum strain GZUIFR - C43 - 1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene complete sequence; and internal transcribed spacer 2, partial sequence.
Sample ID: EU004812.1

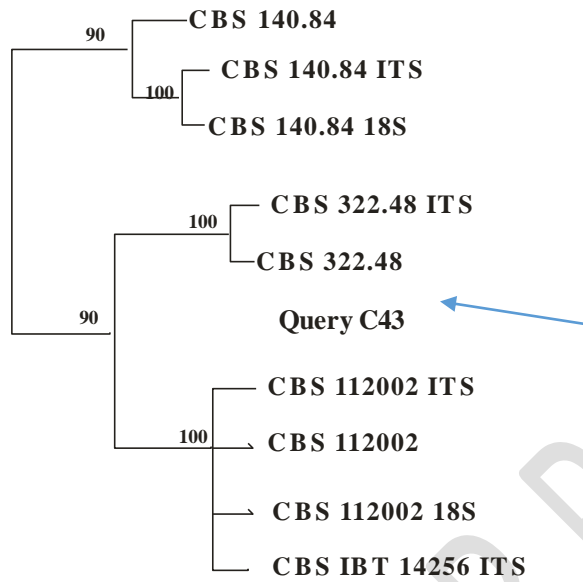


Figure 8: Phylogenetic tree of *Trichophyton violaceum* isolated from scalp lesion of a 22 year – old male staff working in school A.

Trichophyton verrucosum CBS 645.80 ITS region; from TYPE material
Sample ID: NR_147423.1

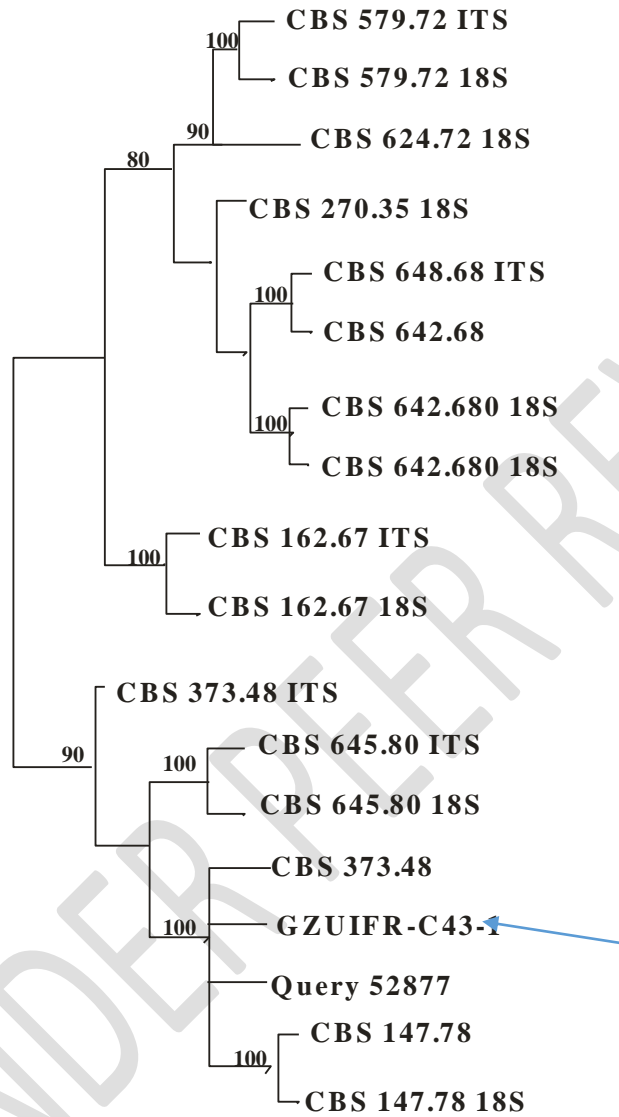


Figure 9: Phylogenetic tree of *Trichophyton verrucosum* isolated from scalp lesion of a 6 year – old male pupil attending school O.

Trichophyton mentagrophytes strain GZUIFR - C43 - 1 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence. Sample ID: EU004812.1

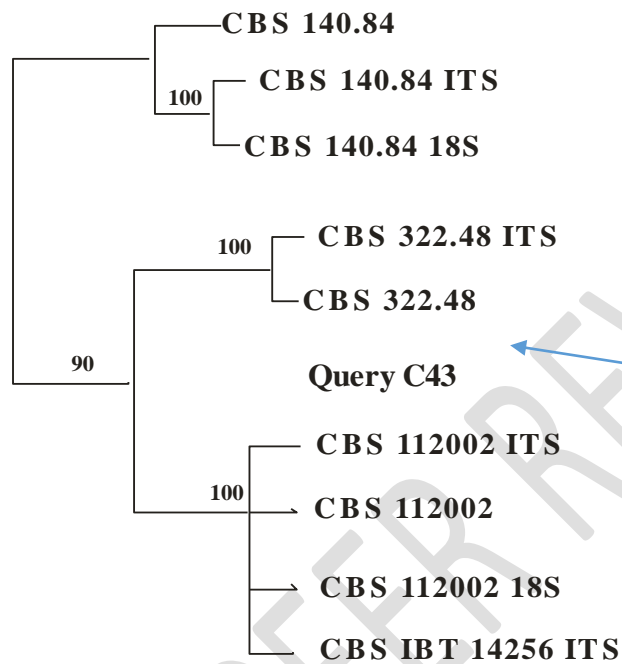


Figure 10: Phylogenetic tree of *Trichophyton mentagrophytes* isolated from scalp lesion of a 13 year – old female pupil in school K.

Epidermophyton floccosum strain CBS 5147 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and larger subunit ribosomal RNA gene, partial sequence.
 Sample ID:

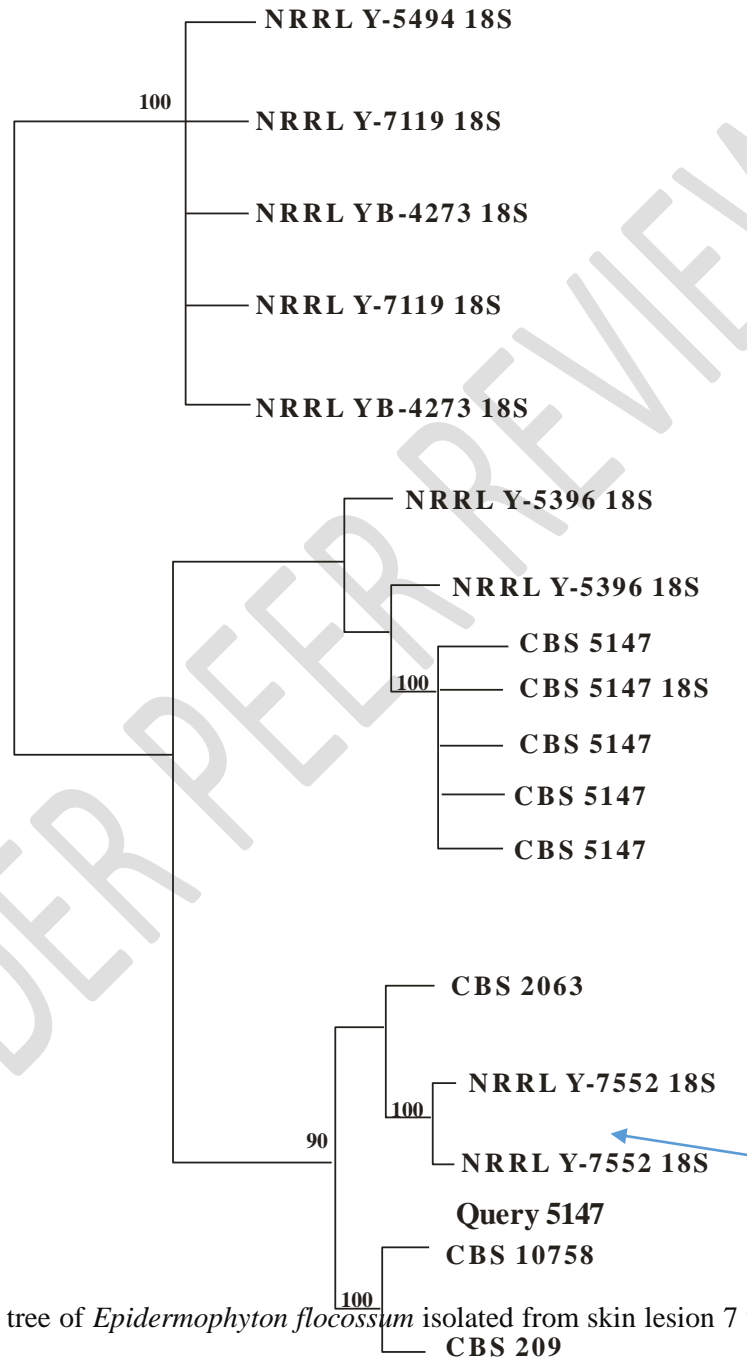


Figure 11: Phylogenetic tree of *Epidermophyton floccosum* isolated from skin lesion 7 year – old female pupils attending school B.

4. DISCUSSION

Development in the application of nucleic acid amplification technology has enhanced the quality of dermatophyte detection. Several nucleic acid-based molecular methods have been developed to detect fungi from clinical specimen targeting 18SrDNA, ITS1 and ITS 2 regions, 5.8SrDNA and 28SrDNA. Therefore, in the present study, two targets of the fungal genome – the ITS region and 18SrDNA – were chosen as they have cleavage sites that could be of value for application of RFLP (restricted fragment length polymorphism) on the amplified product to detect dermatophytes. Out of 1000 samples collected, consisting of 500 samples each from Anambra East and Ayamelum L.G.A of Anambra State; 320 were both culture and KOH positive out of this 12 of the positive samples were subjected to molecular typing and all were positive. This agrees with the work of (13). PCR and RFLP is rapid and results can be obtained within 8 – 9 hours from samples directly from DNA extraction to reporting by electrophoresis. This technique is more accurate, sensitive and from source detection and also for epidemiology identification of the disease.

Phylogenic tree indicates that ancestral image gave rise to all organism on the tree. A branch point indicates where the two lineage diverged. A lineage that evolved early and remains unbranched is a basal taxon. When two lineage stem from the same branch point, they are sister taxa. It also helps in knowing the evolutionary history of organisms or groups of organisms. It shows "How and when other branches of phylogenic tree have evolved from the main stock. It discloses the time of origin and subsequent evolution from simple to complex.

From the molecular DNA and protein extractions of samples from pupils and staff of Anambra east and Ayamelum L.G.A, there was relationship between the *Tricophyton.tonsurrans* isolated from the scalp lesion of 26 years old male staff working in school H, and scalp region of a 8 year old female pupil attending school E, and groin lesion of a 53 years old female staff teaching in school L in the same area. There was also a very close relationship between *Microsporum audouinii* isolated from scalp region of a 14 year old male pupil in school K and the one isolated from skin lesion of a 39 year old male staff at school N, is of the same ancestors with the one isolated from the scalp lesion of a 12 years old female pupil at school A. *Microsporum canis* isolated from a skin lesion of a 7 year old female pupil in school H has no ancestral relationship with other *Microsporum* species isolated. *Tricophyton mentagrophytes* isolated from the scalp lesion of a 13 year old female pupil attending school K also has no ancestral relationship with other *Tricophyton mentagrophytes* in line with (14). From all the comparisons made from the isolates the phylogenic trees was used to estimate the relationships among the species represented by these sequences.

5. CONCLUSION

Dermatophytic infections are common contagious disease. Primitive diagnosis of dermatophytosis can be done by KOH mount and culture, which takes longer time to report and cannot differentiate at the level of genus and species level. Results from this study indicate that PCR – RFLP may be considered as gold standard for the diagnosis and confirmation of source of infection of dermatophytosis and can aid the clinician in initiating prompt and appropriate antifungal therapy.

In conclusion, From the 12 isolates that went through molecular typing using the DNA and protein sequences, the phylogenic trees showed a very close genetic relatedness between *Tricophyton tonsurans* isolates from different pupils and schools. This also include *Microsporum audouinii* and *Tricophyton rubrum*, *Microsporum canis* and *Tricophyton metagrophytes*. There was no ancestral relatedness between *Microsporum canis* and *Tricophyton metagrophytes*.

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