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Phylogenetic Tree Analysis of Dermatophytes using Sequence of the 18S rRNA Gene ITS Region

Neeran Obied AL-Amari[1*] Noor Jasim Mohammed[2]
1.College of Pharmacy/ Al-Qadisiyah University/Diwaniyha/Iraq
2.College of Science/ Al-Qadisiyah University/ Diwaniyha/Iraq
*Email of the corresponding author: Neranjasim@yahoo.com

Abstract
Dermatophytes usually identified on the basis of isolation patterns together with conidial morphology, and some-times with physiological characters, such as the hair perforation and urease tests. In some cases, morphological identification can be difficult because there is great variation and polymorphism among isolates of the same species. Recent, genotyping approaches have proven to be useful for solving problems of dermatophyte classification. Molecular identification such as ITS sequencing could become part of the diagnostic for dermatophytosis, In present study, results show genetic analysis of the tree shows the evolutionary relationships between species, which helps in the diagnosis of suspected species, especially *T.rubrum and M.canis* .
Keywords: phylogenetic tree, *T.rubrum , M.canis* , PCR, Dermatophytes.

Introduction:

Exposed the skin to microbial infection caused by different types of microorganisms, whether on the skin naturally with opportunistic nature to cause skin infections, or that come from the environment , such as Dermatophytes group (Weinstein & Berman, 2002), where is the skin more members of the body exposed to the external environment, and has the means of many skin protection from various injuries although it contains many numbers of microorganisms that occur naturally in it (Odell, 1998). From the means important of the skin protect that inhibit the growth of different pathogens are relatively dry skin and low pH value (4-5 = pH) as well as the large number of fatty secretions (Finch, 1988). There are factors that make the skin at risk significantly, including: inhibition of the immune, diabetes, burns and wounds (File & Tan, 1991). Skin infections are considered common infections in humans for a long time, as 10-15% of world's population are exposed to skin infection (Matsumoto, 1996)), as skin fungus to the family of Arthrodermataceae that cause skin infections known Tinea or cutaneous mycosis (Dermatophytosis) in humans and animals (Rosenthal, 1998). fungi possess significant advantages of being keratinophilic and keratinolytic (Simpanya & Baxter, 1998) but does not have the ability to penetrate deep tissue under of Stratum corneum layer, where most of them unable to live in a higher temperature (35 ˚C) as well as the presence of inhibitory factors in serum and body fluids which inhibit the hydrolyzed keratin enzyme (Brooks *et al*., 2001) The fungal infections of skin occur by one of the species *Trichophyton* , *Epidermaphyton and Microsporum* (Habif, 1996)). , which affects millions of people in the world and that healthy people or who suffer from Immunosuppression are at risk of infection(Pierard *et al*., 1996) The processes of speciation in dermatophytes, especially anthropophilic dermatophytes, appear to have been anomalous, and species themselves may be difficult to define (Gräser. *et.al*.,2006) In the present study we determined the phylogeny of the group of dermatophytes, including the genera *Trichophyton*, *Microsporum*, and *Epidermophyton*, and identified the species using the base pair sequences of ITS1**.**

Material &Methods:

Clinical specimens: One hundred clinical specimens were collected from patients with skin infections who reviewed the advisory Dermatology in Al- Diwaniyah Teaching Hospital for the period from October / 2013 to April / 2014. 72 samples give positive for direct microscopic examination and 65 samples give positive for culture method. These included 29 samples of skin scarping, 24 samples of hair and 19 samples nails. These samples transferred to the laboratory for testing.

Examination of specimens:

Direct examination: Examined the hair and skin samples according to (Koneman *et al*.1978) by using 10% KOH and heating gently.

Cultivation of samples: The samples were cultured on Sabuaroad dextrose agar containing of chloramphenicol (250 mg / ml) to prevent the growth of saprophytic bacteria, and then was added cycloheximid (0.05 mg / L) to prevent contaminated with saprophytic fungi. This incubated at temperature (2 ± 30) ˚m for 21 days.

Diagnosis by routine procedure:

This conducted according of conidial morphology, and physiological characters, such as the hair perforation and urease tests and the genus was identified according of (Koneman *et al*., 1978; Kwon-Chung & Bennett, 1992).

Diagnosis using PCR assay:
Use this diagnosis to confirm the routine diagnosis and to emphasis the identifying of the isolates that suspected diagnosis of it. PCR assay was using primers special of the gene of 18S rDNA gene were used two types of special prefixes, the first (18S rRNA gene ITS region) that diagnosis genus*Trichophyton* and second (18S rRNA gene ITS region's) diagnosis of genus *Microsporum* where primers designed in this study from Gen bank NCBI by using Primer3plus program to design primers and private screening in the PCR, primer and equipped by the Korean Bioneer Corporation.
Analyzing the results of the PCR assay: Was conducted gel electrophoresis using gel Agarose gel 1.5% and according of (Sambrook *et al.*, 1989) so as to read as a result of the interaction of PCR product.
DNA sequencer method: Was a method of DNA sequencing to identify the *Trichophyton and Microsporum* species that diagnosed by phenotypic character and PCR assay through a Phylogenetic tree analysis of gene ITS1 small subunit rRNA gene, which in the beginning was to hold the PCR reaction-long (411bp PCR product) for the fungus *Microsporum* and *(*397bp PCR product) for the fungus *Trichophyton*. Then been sent to the output of the PCR reaction Macrogen company in South Korea and to conduct DNA sequencing using the device AB DNA sequencing system.
Results:
Diagnosis by routine procedure:
Results of diagnosis by using macro and micro features, show that identified two genus *Trichophyton* sp. and *Microsporum* sp. and four species : *Trichophyton rubrum , T.mentagrophytes , T.schoenlenii , M.canis* .Also results of cultivation appear some isolates could not identified according routine procedure. This results accordant with AL-Kafaji (2001) and Abed Ali(2014).which reported that these species abundant in clinical specimens .
Diagnosis by PCR:
After diagnosed the fungal species that isolated by routine methods based on determining phenotypic criteria using taxonomic keys as shown previously and to ensure the validity of diagnosis was used two types of primer, the first (18S rRNA gene ITS region) diagnosis of *Trichophyton* and second (18S rRNA gene ITS region) of the diagnosis of *Microsporum* primer were designed in this study from the Gen bank NCBI using Primer3plus program to design primers for PCR to examine the results of the examination were as follows:

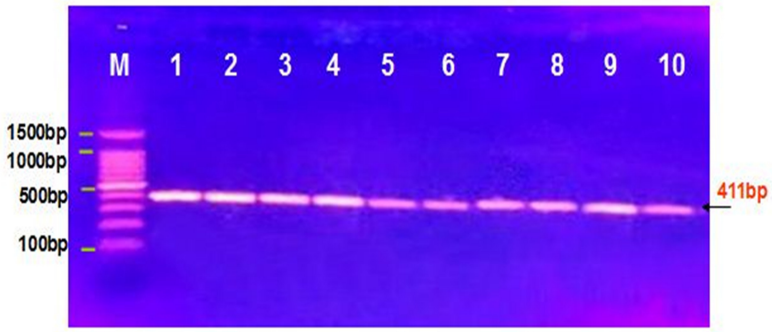
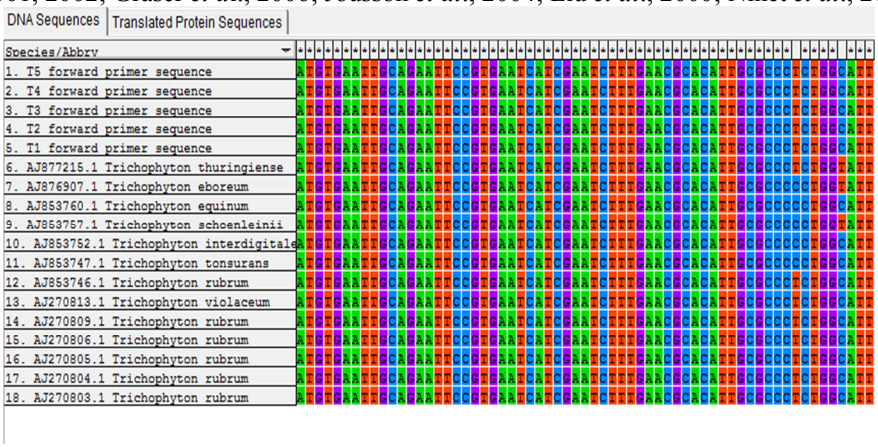


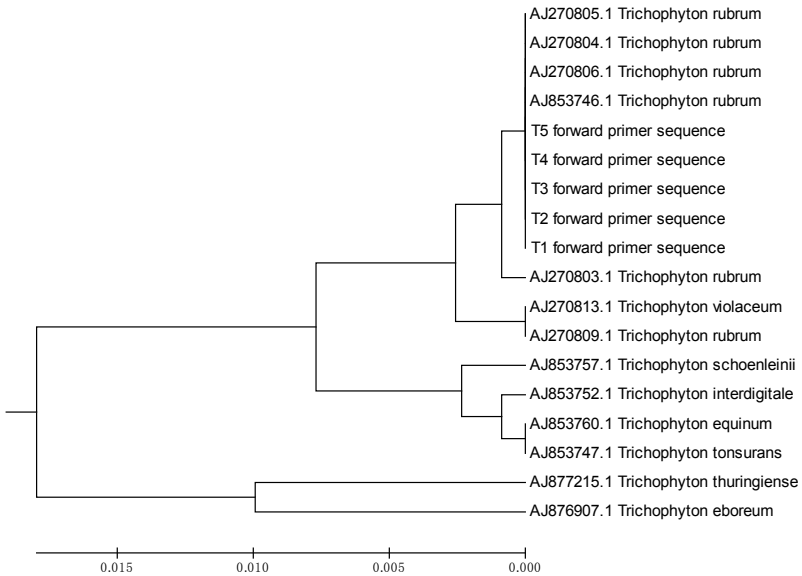
Figure (1)Electrophorsis of DNA on agarose gel (1.5%) for hours to isolates Trichophyton spp. (1-10) w here: M DNA Ladder (100-1500) base pair

Figure(2) Electrophorsis of DNA on agarose gel (1.5%) for hours to isolates Microsporum sp (1-10) where: M DNA Ladder (100-1500 bp)
after completing the PCR assay, been sent screening products to Gen Bank site to make sure the fungus species

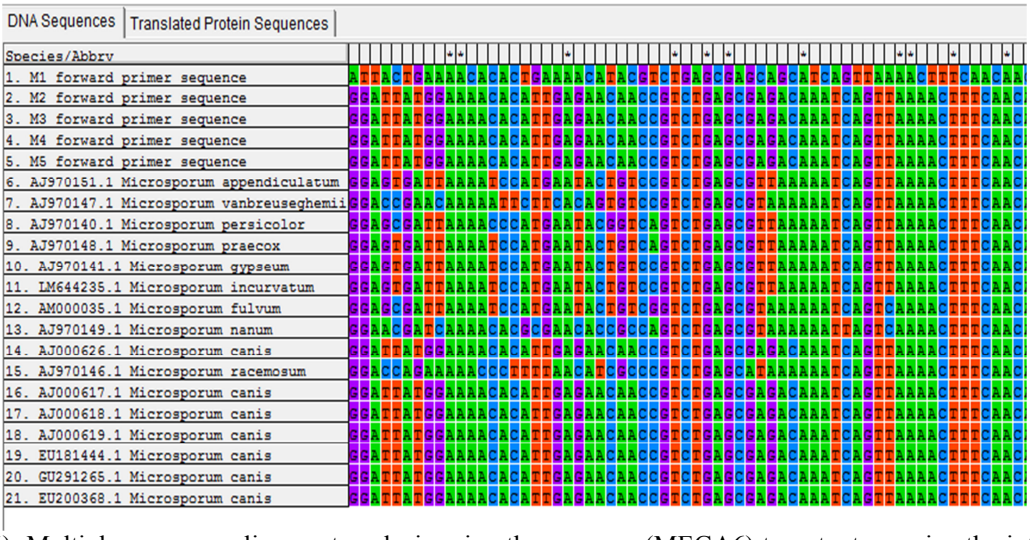
by comparing it with the species recorded at this location as in the figure (3, 4,5,6) and using MEGA6 program were used genetic analysis of tree type UPGMA tree (Weighted Pair Group Method with Arithmetic Mean) where the results of the analysis showed a clear convergence of isolates fungus spp. Local isolates with local *Trichophyton rubrum*, compared with the rest of the other species that appear in the analysis of the genetic tree. As for the type of *Microsporum sp*. have been diagnosed through genetic analysis of tree type UPGMA tree (unweight Pair Group Method with Arithmetic Mean) where the results of the analysis showed a clear convergence of isolates fungus spp. Local us *Microsporum canis* compared with the rest of the other species that appear in the analysis of the genetic tree. Genetic tree used to know every link own species with a particular species of genus to be determined routine (Emilie ., 2007).In some cases the diagnosis phenotypic and microscopic difficult or uncertain because of the great diversity or polymorphism of the same type, to identify useful genetic pattern in the classification of fungi and enhances the accuracy and speed of initial diagnosis (Faggi *et al*., 2001, 2002; Graser *et a*l., 2006; Jousson *et al*., 2004; Liu *et al*., 2000; Ninet *et al*., 2003).



Figure(3): Multiple sequence alignment analysis using the program (MEGA6) to output examine the interaction sequence polymerase PCR gene ITS1 small subunit rRNA gene in the *Trichophyton,* where notes the similarity clear alignment sequence rules for ITS1 gene)) in isolates fungus spp. Local domestic isolates with *Trichophyton rubrum* recorded in Gen Bank site



Figure(4): Phylogenetic tree analysis using the program (MEGA 6) where the use of genetic tree analysis of the species (UPGMA tree) (Unweight Pair Group Method with Arithmetic Mean) where the results of the analysis showed a clear convergence of isolates fungus spp . Local domestic isolates Trichophyton *rubrum*, compared with the rest of the other species that appear in the analysis of the genetic tree.



Figure(5): Multiple sequence alignment analysis using the program (MEGA6) to output examine the interaction sequence polymerase PCR gene ITS1 small subunit rRNA gene in the *Microsporum*, where notes the similarity clear alignment sequence rules for ITS1 gene in *Microsporum* isolates with *Microsporum canis* recorded in Gen Bank site

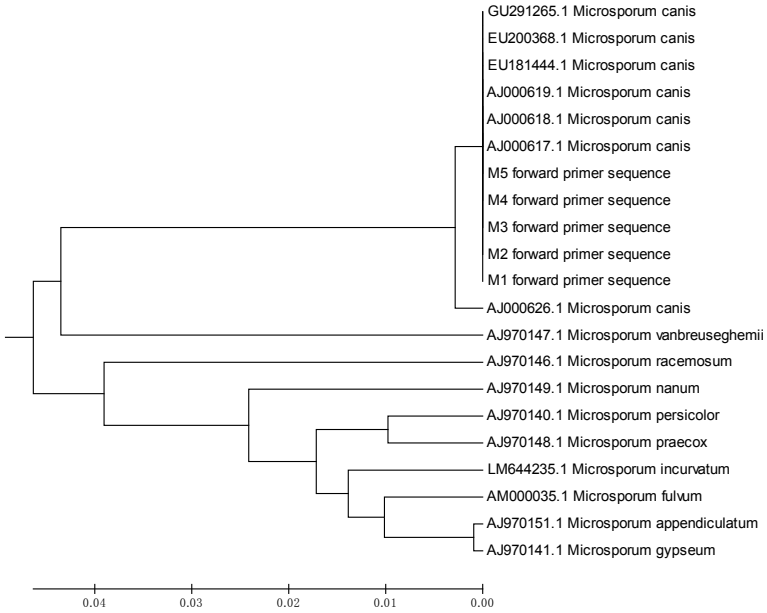


Figure (6.): Phylogenetic tree analysis using the program (MEGA 6) where the use of genetic tree of the species of analysis (UPGMA tree) (Unweight Pair Group Method with Arithmetic Mean) where the results of the analysis showed a clear convergence of Local us *Microsporum canis* compared with the rest of the other species that appear in the genetic tree analysis.

Conclusions

From our results we can be conclusions that morphological and physiological characteristics useful in diagnosis of species of dermatophytes and variation and pleomorphic in these species ,makes identification of it unsatisfactory .Also , sequence analysis of the ITS region and phylogenetic tree has to be useful tool to identification of species of dermatophytes.

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