Iraqi Journal of Biotechnology, 2023, Vol. 22, No. 1, 220-228

Molecular Identification of *Tinea spp*. Causing *Tinea* Disease using ITS Sequencing Analysis

Qadisiyah H. Hashoosh, Alaa M. AL-Araji

Department of Biology, College of Sciences, University of Baghdad

Received: April 16, 2023/ Accepted: May 28, 2023/ Published: June 4, 2023

Abstract: *Tinea* is a group of keratinophilic fungal infections caused by dermatophytes that invade the skin, hair, and nails. As the conventional methods for dermatophytes detection are time-consuming or lack enough specificity, accurate diagnostic methods such as molecular techniques are required for precise identification and differentiate between two closely related species of dermatophytes. To disclose the useful role of DNA sequencing in the recognition of dermatophytes using the ITS region and comparing the current local isolates with those provided by the NCBI. The study aims to diagnose the dermatophyte isolates causing ring worm diseases using the inter transcribed spacer (ITS) region and find its phylogenetic analysis. one hundred ten (skin scraping, hair clippings and nails) samples were gathered from patients with dermatophytosis. All specimens were being microscopically inspected and then cultured on sabouraud's dextrose agar (SDA) with cycloheximide, PCR with ITS1 and ITS4 primers was used to confirm the positive dermatophytes' cultures. The PCR results were sequenced, and the rRNA database was used to do analysis on the sequences and verify the homogenic data of the microorganisms (NCBI). Substitutions in ribosomal RNA gene were found 37 isolates from dermatophytes and 8 isolates of them contained variations of the percentage of conformity with the gene bank 99% and they were registered with the gene bank and wrote down the new code that was registered in the table and in front of the heterogeneous sample. *Trichophyton mentagraphytes* recorded the highest frequency from cases. *Trichophyton simii* recorded the highest genetic several transitions, transversion, deletion, and insertion mutation when compared to other globally known. The phylogenic trees showed clear genetic interfere. It was concluded, demonstrate that the ITS region developed in this study can be used for detecting the *Microsporum, Trichophyton, and Epidermophyton*.

Keywords: ITS region, Dermatophytes, Phylogenic tree, insertion mutation, Iraq.

Corresponding author: (Email: qadesia.halal1102a@sc.uobaghdad.edu.iq).

Introduction

Based on their asexual properties, the three genera *Microsporum*, *Trichophyton*, and *Epidermophyton* make up the phylum Ascomycota, class Euascomycetes, order Onygenales, and family *Arthrodermataceae* of dermatophytes. Infections in the skin, feathers, hair, hooves, horns, claws, and nails are brought on by these substances (1). Phenotypic methods can define dermatophyte isolates to genus/species depending on the appearance of colonies, microscopic examinations, and biochemical assessments such as growth patterns on urease and *Trichophyton* agars (2). Depending on morphological identification of dermatophytes may be difficult or unclear because there are overlying characters between species (3). Molecular biology techniques are modified to identify dermatophytes and can be considered effective compared to conventional tests (4). In order to offer quicker and more reliable alternatives for dermatophyte identification, molecular techniques have been developed. These techniques include RFLP analysis (5), DNA hybridization, PCR fingerprinting (6) gene-specific PCR (7), the chitin synthase-encoding

gene (8), and sequencing of the large subunit rRNA gene (9). For accurate specification, genome of dermatophytes is analyzed. The polymorphism of the internal transcribed spacers (ITS1 and ITS2) next to the DNA sequence encoding rDNA is very precise and dependable for differentiating species of dermatophytes (3,4).The study aims to diagnose the dermatophyte isolates causing ring worm diseases using the ITS region and find its phylogenetic analysis.

Materials and methods

Samples collection

One hundred ten clinical samples (nails, skin scrapings and hair clippings) were gathered from patients attending the Dermatology Department of AL-Emamain AL-Kadhumain Teaching Hospital and AL-Zahraa Consulting Center for Allergy and Asthma/ Baghdad between January 2021 and September 2021 regardless of age and sex of patients. Each specimen was split into two pieces, with one part being microscopically inspected and the other being grown on SDA with cycloheximide and the second part extracted the DNA directly from (skin scraping nails and hair clippings).

Primary fungal examination

The demonstration of fungal elements (branching and septate hyphae and spores) in hair, skin, and nails by direct microscopic examination is the basis for the diagnosis of infection. The samples were laid out on a clean slide that had been gradually heated (30Co) for about 5–10 minutes, flooded with drops of 10% KOH, and then the slide was allowed to cool before the cover slip was added and viewed with a low-power (10X) and high-power (40X) microscope.

Culturing of samples

The samples were grown on containing Sabouraud's dextrose agar containing cyclohexamide and chloramphenicol medium (10). Each skin scrape, nail, and hair clipping specimen was inoculated by a sterile scalpel into Petri dishes and then, incubated at 28±2 °C. Cultures were first examined after 7 days, then twice weekly for at least 3-4 weeks before being considered negative.

Macroscopical check of the cultures

Examinations are made of the colony's rate of growth, color, texture (fluffy, cottony, wiry and suede-like), surface topography (folded, flat, rugose, plicate), reverse side (medium pigmentation), Human pathogenic fungi were identified in this investigation according to (11).

Examining cultures under a microscope B1-Wet mount preparation

A little part of the colony was carefully teased out on the slide using a flamed bent inoculating needle or straight wire and stained with a drop stain (lactophenol cotton blue). A cover slip was applied with light pressure and examined at low and high power (12).

DNA extraction directly from (skin scraping nails and hair clippings)

DNA extracted from skin rubbings, nails, and hair clippings directly, using FavorPrep Fungi/ Yeast Genomic DNA Extraction Mini Kit (Cat. No.: FAFYG 001).

Extraction of nucleic acid for positive dermatophytes cultures

The "Genomic DNA extraction Kit" was used to isolate the genomic DNA from the fungal growth in accordance with the ABIO pure Extraction methodology, and the DNA was then kept at -20°C until use.

Detection of ITS region by conventional PCR

PCR was performed on the PCR system with a reaction volume of 50 µL, including 25 µL of Maxter Mix, 4

µL of each primer pair HSD3B1 as in (Table 1) 13 µL of nuclease free water, and 4 µL of DNA. The amplification software was as follows: initial denaturation at 95°C for 5 min; denaturation at 95 °C for 30 sec., annealing at 55°C for 30 sec., extension 1 at 72°C for 30 sec; and extra extension at 72°C for 7min for 30 cycles. The PCR products were exposed to 1.5% agarose gel electrophoresis at 70 volt for 30 min using the UV- trans-illuminator was used to visualize DNA bands.

Table (1): Primers for amplifying the ITS region

Primer Name	Sequence 5`-3`	Annealing temp. (°C)	Product size (bp)
ITS1	TCCGTAGGTGAACCTGCGG	55	Variable
ITS4	TCCTCCGCTTATTGATATGC		

Sequencing the PCR amplicons of ITS region

Korea's Macrogen Corporation delivered PCR products for Sanger sequencing utilizing the ABI3730XL automated DNA sequence. The results were emailed to us, and we used specialized software to analyze them. and following the amplification of fungi's RNA ribosomal, the validation of microorganisms' homogenic data using the rRNA database (NCBI).

Phylogenic tree

Phylogenetic trees were constructed from unambiguously matched sequences using the neighbor-joining (NJ) technique and the Tamura-Nei parameter as the substitution model as implemented in the MEGA6 program.

Results and discussion

Primary fungal examination

The results showed that 58 specimens out of the 110 tested positive for fungi giving a prevalence rate of 52.7%. The number of collected specimens consisted of 64 (58.2) skin scrapes, 24 (21.8%) nails, and 22 (20%) hair clippings specimens as table (2).

Table (2): Prevalence of human pathogenic fungi among clinical specimens.

Type of specimens	Number of specimens	Number positive	Number negative	Prevalence rate (%)
Skin	64	36	28	58.2
Nail	24	10	14	21.8
Hair	22	12	10	20
Total	110	58	52	100

Also, the result for 58 clinical specimens revealed that *Tinea corporis* was a higher prevalence at about 40% (23 specimens) followed by *Tinea unguium* at 21% (12 specimens) and Tinea capitis at 14% (8 specimens) compared to other types of *Tinea* , as shown in figure (1). This study backs up previous results of dermatophytosis in Nigeria, *Tinea corporis* was reported as the most popular dermatophyte infection (13). In another study in Mali, Tinea corporis prevalence was 21% (14). Unlike prior research, (15) found a high prevalence of *Tinea pedis*.

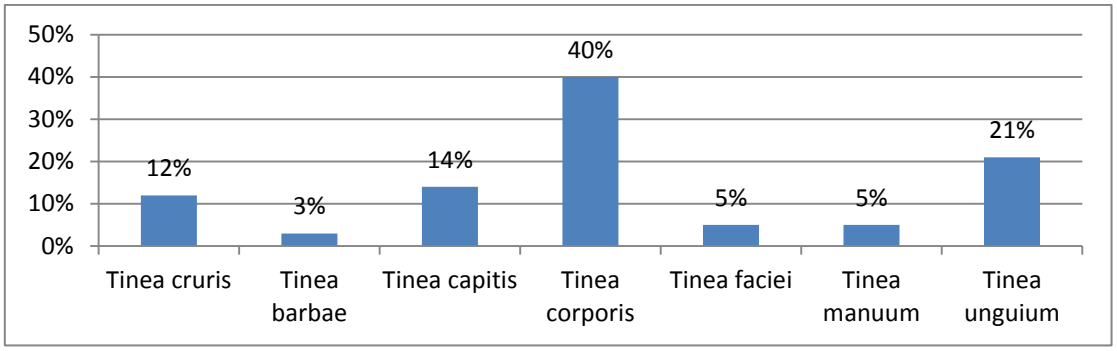

Figure (1): Distribution of cases, according to clinical manifestations of dermatophytes.

The results of the direct examination and the morphological diagnosis (culture) were identical, including fife-eight, positive samples, thirty-seven of which were dermatophytes as shown in figure (2), and the results were identical to the molecular diagnosis. *Trichophyton mentagrophytes* isolates were the most common fungus isolated, indicating a significant prevalence of this fungus causing dermatophytosis as shown in figure (2). The other fungus isolates, as shown in figure (3).

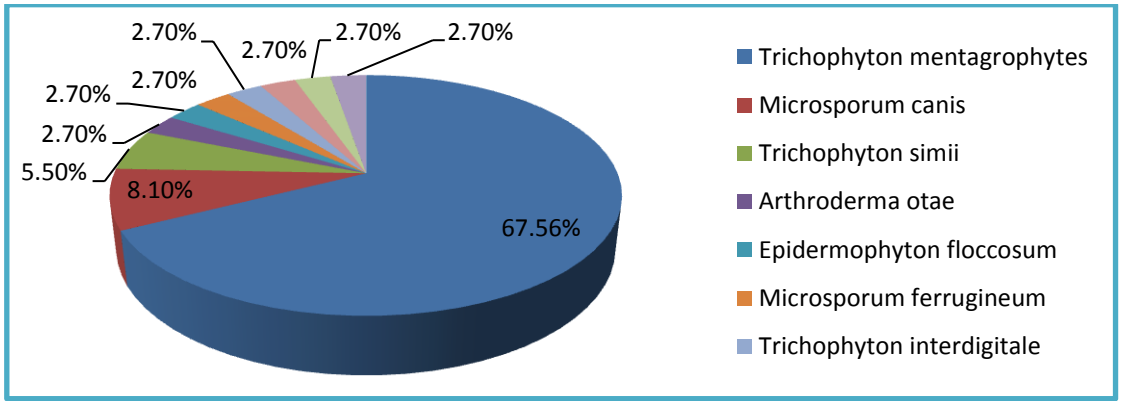

Figure (2): Dermatophytes fungi isolated from various cases of tinea patients

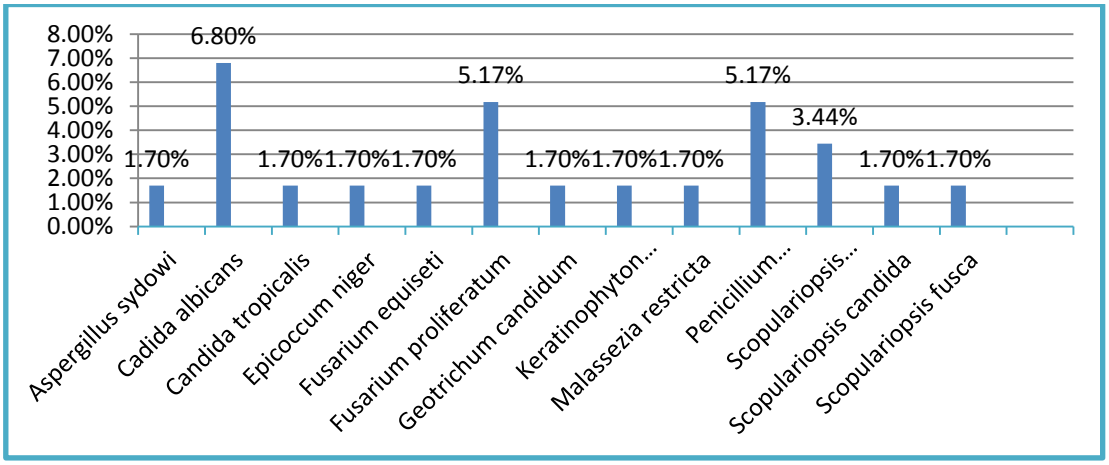

Figure (3): different species of opportunistic fungi isolated from various cases of tinea patients.

Results of PCR for detection the amplicon of ITS region

Gel electrophoresis revealed bands of extracted nucleic acid from ITS region (figure 4).

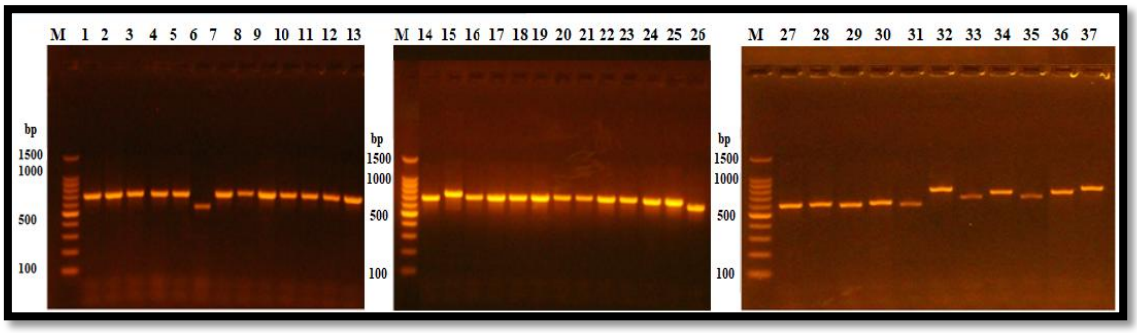

Figure (4): Gel electrophoresis of ITS region of rRNA (1.5% agarose gel with Ethedium Bromide at 7 volte for 1 hour) M: 100-1500 bp DNA ladder

Sequencing analysis of ITS region

Geneious software was used for the sequencing investigation, which revealed that the small subunit ribosomal RNA gene's nucleotide sequence differed from other internationally recognized genes. They were insertion, deletion, transversion, and transition. These alterations are detailed in table (3).

Table (3): Genetic variation small subunit of ribosomal RNA Gene (ITS region).

Small subunit ribosomal RNA gene (ITS region)							
No.	**Type of substitution**	**Location**	**Nucleotide**	**Sequence ID with compare**	**Source**	**Identities**	**New Accession**
1	--------	-----	--------	MT371235.1	*T. mentagrophytes*	100%	OP821432.1
2	--------	-----	--------	MT371235.1	*T. mentagrophytes*	100%	OP821433.1
3	--------	-----	--------	MT371235.1	*T. mentagrophytes*	100%	OP821434.1
4	--------	-----	--------	MT371235.1	*T. mentagrophytes*	100%	OP821437.1
5	--------	-----	--------	MT371235.1	*T. mentagrophytes*	100%	OP821438.1
6	--------	-----	--------	MT633048.1	*M. canis*	100%	OP821441.1
7	--------	-----	--------	MT371235.1	*T. mentagrophytes*	100%	OP821443.1
8	--------	-----	--------	MT371235.1	*T. mentagrophytes*	100%	OP821444.1
9	--------	-----	--------	MT371235.1	*T. mentagrophytes*	100%	OP821446.1
10	--------	-----	--------	MT371235.1	*T. mentagrophytes*	100%	OP821447.1
11	--------	-----	--------	MT371235.1	*T mentagrophytes*	100%	OP821450.1
12	--------	-----	--------	MT371235.1	*T. mentagrophytes*	100%	OP821453.1
13	--------	-----	--------	MT345059.1	*M. ferrugineum*	100%	OP821455.1
14	--------	-----	--------	MH862931.1	*M. canis*	100%	OP821456.1
15	--------	-----	--------	MT345059.1	*T. mentagrophytes*	100%	OP821457.1
16	--------	-----	--------	MT345059.1	*T. mentagrophytes*	100%	OP821458.1
17	--------	-----	--------	MT345059.1	*T. mentagrophytes*	100%	OP821459.1
18	--------	-----	--------	MT345059.1	*T. mentagrophytes*	100%	OP821460.1
19	--------	-----	--------	MT345059.1	*T. mentagrophytes*	100%	OP821461.1
20	--------	-----	--------	MT371235.1	*T. mentagrophytes*	100%	OP821463.1
21	--------	-----	--------	MT371235.1	*T. mentagrophytes*	100%	OP821464.1
22	--------	-----	--------	MT371235.1	*T . mentagrophytes*	100%	OP821466.1
23	Transition	122	C\T	OP391648.1	*T. simii*	99%	OP821467.1
	Transition	157	T\C				
	Transition	582	T\C				
	Transition	583	T\C				
24	--------	-----	--------	MT371235.1	*T. mentagrophytes*	100%	OP821469.1
25	Transition	292	T\C	AB193667.1	*M. canis*	99%	OP821470.1

					(Arthroderma otae)		
26	Transition	517	C\T	OP391648.1	*T. simii*	99%	OP821471.1
27	--------	-----	--------	MT371235.1	*T mentagrophytes*	100%	OP821477.1
28	--------	-----	--------	MT371235.1	*T. mentagrophytes*	100%	OP821478.1
29	--------	-----	--------	MT371235.1	*T. mentagrophytes*	100%	OP821479.1
30	--------	-----	--------	MT371235.1	*T. mentagrophytes*	100%	OP821480.1
31	Transition	290	C\T	MH862931.1	*M. canis*	99%	OP821483.1
32	Transvertion	145	T\G	MN808779.1	*T. quinckeanum*	99%	OP821484.1
	Transition	478	C\T				
33	--------	-----	--------	MT497455.1	*E. floccosum*	100%	OP821485.1
34	Transvertion	17	T\A	MZ574566.1	*T. verrucosum*	99%	OP821486.1
	Transvertion	167	A\C				
	DELATION	225	A\-				
	DELATION	269	T\-				
35	INSERTION	11	-\G	KF437402.1	*T. rubrum*	99%	OP821487.1
	Transition	162	C\T				
	Transvertion	658	G\T				
36	Transition	189	G\A	MK312877.1	*T. mentagrophytes*	99%	OP821489.1
	Transition	633	C\T				
37	--------	-----	--------	MT633091.1	*T. interdigitale*	100%	OP821449.1

Substitutions in ribosomal RNA gene were found 8 isolates (*A. otae* (1), *M. c (1), T. m (1), T. q* (1), *T. s* (2), *T. r* (1), *T. ve* (1) of them contained variations of the percentage of conformity with the gene bank 99% and they were registered with the gene bank and wrote down the new code that was registered in the table and in front of the heterogeneous sample in this study. According to Table 2, the ribosomal RNA gene of *T. m* isolates showed transition mutation at G/A and C/T in ribosomal RNA gene , but *T. s* are 99% similarity, include transition mutation at C/T and T/C in ribosomal RNA gene. *T.q* are 99 % similarity, include transvertion and transition mutations at T/G and C/T in ribosomal RNA gene. While *T. ve* are 99% similarity, include transvertion at (T/A and A/C) and deletion at (A/- and T/-) in ribosomal RNA gene. But the *T. rubrum* are 99% similarity, include several mutations include insertion at -/G and transition at C/T and transvertion at G/T in ribosomal RNA gene.

The *M. ferrugineum* and *M. c* and *E. floccosum* isolates showed no substitutions but some of them *M. c* (*A. otae*) are 99 % similarity include transition at C/T in ribosomal RNA gene. Generally speaking, these modifications to the ribosomal RNA genes could lead to high levels of antifungal resistance and/or play a supporting role in the development of high levels of pathogenicity in *Trichophyton*, *Microsporum*, and *Epidermophyton*, or the opposite. According to our interpretation, this outcome may have been caused by Indian immigrants who had tinea and who mixed with Iraqis, who then spread the infection. As a result, the co-infection of the different strains causes genetic changes that result in the formation of new strains.

In this study, DNA sequencing was used to analyze the PCR results by maximizing the rDNA gene's ITS region. Through the use of the ITS, it is possible to communicate with phylogenetically related extra-faraway fungal species. The PCR results underwent normal sequencing procedures in South Korea (Macrogene lab). Sequence analysis of the more variable ITS region can be utilized to differentiate between various species of

dermatophytes, which exhibit highly pleomorphic ITS regions within the same genus. It could potentially be used to resolve the phylogenetic relationships within the *Arthrodermatacae* family. The fungal of superficial infections are becoming more widespread in emerging nations such as Iraq and India. A study was conducted in 2018 in Iraq and it proved *T. m* was the most common species (21.7%) isolated among 30 positive dermatophytes (15).

Dermatophytes are the most common causative agents. Due to their ability to devour keratin, they result in a range of clinical symptoms. Dermatophyte skin infections are frequently confused with other non-fungal skin illnesses (16). They are the most frequently isolated species mainly from patients with tinea corporis. The higher frequency with *M.c* and *T. m* may be explained to the direct or indirect contact with domestic animals such as cattle because they are zoophilic fungi and cause many ringworm infections. (17,18). The most common fungal infection occurred in summer season (42.16%), and the most frequent month manifestation of species occurred in August (17%) (19, 20). In other study, ((the number of dermatophytes isolates was 21 which represent (19.09%) of the total cases.

The number of *Candida* isolates was 60 which represent (73.17 %) of the total cases. The number of opportunistic fungal isolates was 29 which represent (26.12%) of the total cases. The result showed that the most common isolates were *C. guillermondii* 19 isolates which represent (31.66 %) of cases, then followed by *C. itermedia* 11 isolates which represent (18.3 %), *C. kyfer* 8 isolates which represent (13.3 %), *A. niger* 17 isolates which represent (58.62%), *A. fumigatus* and *A. flavus* 3 isolates which represent (10.34 %), *Penicillium* 5 isolates which represent (17.24 %), and *Alternaria* 1 isolate which represent (3.44%) (21).

Dermatophytosis can have an impact on one's social, psychological, and vocational well-being. Because there is no vaccination for dermatophytosis, raising knowledge about the disease, prompt diagnosis, and appropriate treatment can assist to halt its spread.

Phylogenic tree analysis of dermatophytes of Iraqi samples

The analysis involved 37 nucleotide sequences. The clades relationship between the dermatophytes isolates were gave a great overlap showed that in figure (4).

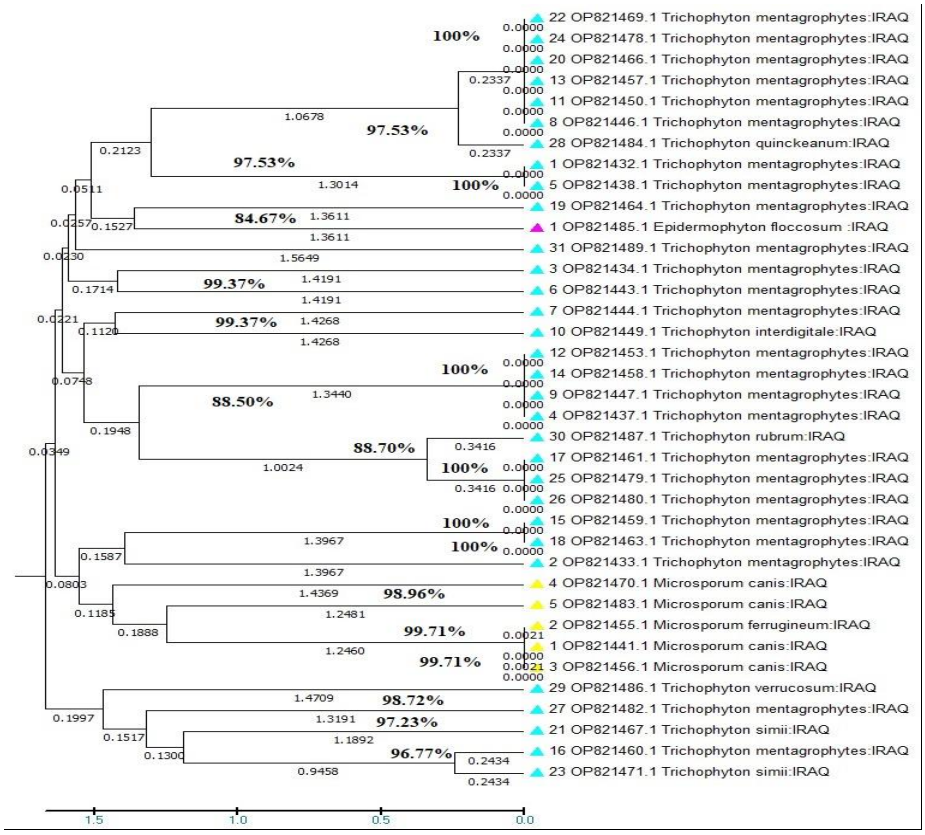

Figure (4): Phylogenic tree of Iraqi sample that explain the ITS region

Conclusion

ITS sequencing is very effective in precise identification of dermatophytes. Dermatophytes should be considered by some prophylactic procedures to limit its transmission.Phylogenic tree analysis has placed most of the isolates from the same species together, showing a high level of genetic relatedness and these were distinct from those from another species.

References

1. Alharbi, K.S.; Joshi, N.; Singh, Y.; Kazmi, I.; Al-Abbasi, F.A.; Alzarea, S.I., *et al*. (2022). Molecular exploration of hidden pleiotropic activities of azoles on dermatophytes in human tinea corporis infection. Journal of Medical Mycology, 32(4):10-16.
2. Diongue, K.; Boye, A.; Bréchard, L.; Diallo, M. A.; Dione, H.; Ndoye, N. W. *et al*. (2019). Dermatophytic mycetoma of the scalp due to an atypical strain of *Microsporum audouinii* identified by MALDI-TOF MS and ITS sequencing. Journal de Mycologie Médicale, 29(2): 185-188.
3. Verrier, J. and Monod, M. (2017). Diagnosis of dermatophytosis using molecular biology. Mycopathologia, 182(1): 193-202.
4. Tartor, Y. H.; Abo Hashem, M. E. and Enany, S. (2019). Towards a rapid identification and a novel proteomic analysis for dermatophytes from human and animal dermatophytosis. Mycoses, 62(12): 1116-1126.
5. Leon Mateos, A.; Paredes-Suárez, C.; Pereiro, J. M. and Toribio, J. (2006). Study of the ITS region in an atypical isolate and comparison with six species of *Microsporum*. Mycoses, 49(6): 452-456.
6. El Fari, M.; Tietz, H. J.; Presber, W.; Sterry, W. and Gräser, Y. (1999). Development of an oligonucleotide probe specific for *Trichophyton rubrum*. British Journal of Dermatology, 141(2): 240-245.
7. Graser, Y.; Kuijpers, A. F. A.; Presber, W. and De Hoog, G. S. (2000). Molecular taxonomy of the *Trichophyton rubrum*

complex. Journal of Clinical Microbiology, 38(9): 3329-3336.
8. Yoshida, E.; Makimura, K.; Mirhendi, H.; Kaneko, T.; Hiruma, M.; Kasai, T. and Tsuboi, R. (2006). Rapid identification of Trichophyton tonsurans by specific PCR based on DNA sequences of nuclear ribosomal internal transcribed spacer (ITS) 1 region. Journal of Dermatological Science, 42(3): 225-230.
9. Ninet, B.; Jan, I.; Bontems, O.; Léchenne, B., Jousson, O., Panizzon, R. and Monod, M. (2003). Identification of dermatophyte species by 28S ribosomal DNA sequencing with a commercial kit. Journal of Clinical Microbiology, 41(2): 826-830.
10. Kwon-Chung, K.J. and Bennett, J.E. (1992). Cryptococcosis. In: Kwon-Chung, K.J. and Bennett, J.E., Eds., Medical Mycology, Lea and Febiger, Philadelphia, 397-446.
11. Tille, P. M. and Forbes, B. A. (2014). Bailey & Scott's diagnostic microbiology (Thirteenth edition.). St. Louis, Missouri: Elsevier.
12. Collee, J.G.; Miles, R.S. and Watt, B. (1996). Tests for the Identification of Bacteria. In: Collee, J.G., Marmion, B.P., Fraser, A.G. and Simmons, A., Eds., Mackie and McCartney Practical Medical Microbiology. 14th Edition, Churchill Livingstone, New York, 131-151.
13. Popoola, T. O. S.; Ojo, D. A. and Alabi, R. O. (2006). Prevalence of dermatophytosis in junior secondary schoolchildren in Ogun State. Nigeria. Mycoses, 49(6): 499-503.
14. Coulibaly, O.; Kone, A. K.; Niaré-Doumbo, S.; Goïta, S.; Gaudart, J.; Djimdé, A. A., *et al*. (2016). Dermatophytosis among schoolchildren in three eco-climatic zones of Mali. PLoS Neglected Tropical Diseases, 10(4): e0004675.
15. Naseif1, T. S.; Mohammed, A.J. and Abbas, H.S. (2020). Molecular Identification of the Dermatophytes Causing Tinea Diseases Using ITS Sequencing Analysis. Medico Legal Update, 20(4): 1641–1648.
16. Sharma, M. and Sharma, R. (2012). Profile of dermatophytic and other fungal infections in Jaipur. Indian Journal of Microbiology, 52(2): 270-274.
17. Aasi, S. R. and Al-Aaraji, A. M. (2018). The inhibitory effect of Trichoderma harzianum CA-07 crude extract against *Trichophyton mentagrophyte* and *Microsporium canis*. Iraqi Journal of Science, 59(3): 1387-1395.
18. Al-Dulaimi, S. A. and AL Bahrani, R. M. (2023). Evaluation of Crude Phenolic Extract of Alhagi graecorum Boiss Plant against Some Dermatophytes Isolated from Patients at Al-Yarmouk Teaching Hospital, Iraqi Journal of Biotechnology, 21(2): 183-206.
19. Mohammad, T.H. and Habeb, K.A. (2014). Epidemiological Study of Keratinophilic Fungi in Baghdad Swimming Pools. Baghdad Science Journal. 11(3): 1122–1129.
20. Kadhum, H.H. and Abood, Z. H. (2022). *Staphylococcus aureus* Incidence in Some Patients with a Topic Dermatitis in Baghdad City, Iraqi Journal of Biotechnology, 2022, 21(2): 13-20.
21. Hasan, A. M. and Abdul Majeed, S.M. (2017). Isolation and identification of opportunistic fungi from patients with different types of leukemia in Baghdad province. Iraqi Journal of Biotechnology, 16(3): 216-22.